Quantitative modelling of the response of earthworms to metals

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人總是在圖安穩的同時有一顆飛揚的心…

Life is not easy for any of us. But what of that?
We must have perseverance and above all confidence in ourselves.
We must believe that we are gifted for something and that this thing must be attained.
Marie Skłodowska-Curie

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Chapter I

General Introduction

1.1 Soil system and metals

Soil is a crucial component of the earth's ecosystem. As the largest biodiversity reservoir on our planet, soil provides habitat for billions of organisms, including bacteria, fungi, protozoa, nematodes, earthworms, arthropods, plants, et cetera (Baskin, 2005). Soil biodiversity underpins various ecosystem processes and functions which deliver benefits to mankind (ecosystem services) (Blouin et al., 2013). Human activities are increasingly causing degradation and impoverishment of soil and decline in biodiversity which in turn threatens to diminish the capacity of the earth to sustain us. This creates a sense of urgency for us to prevent biodiversity loss, to protect soil functioning and to maintain sustainability of ecosystems. Soil is a dynamic and heterogeneous environment consisting of solid, liquid, and gas phases. The solid phase includes organic material and mineral particles, the liquid phase contains dissolved organic matter and dissolved nutrients, and the gas phase is composed of volatile organic substances and various gases. Soil textures are classified according to the relative proportions of sand (0.05mm - 2mm), silt (0.002mm - 0.05mm) and clay (< 0.002mm) present in a soil (Davis and Bennett, 1927). Various soil constituents show a great capacity to adsorb and retain metals (Van Leeuwen and Hermens, 2007). As a result, soil may act as a sink for the metals released into the environment.

Sources of metals

Metals are naturally occurring components in the environment, with their occurrence primarily in rocks. The release of metals to soil is facilitated by weathering of parent rock and atmospheric deposition (Reeder et al., 2006). Consequently, natural concentrations of metals in soils are strongly correlated to the varied distribution of rock types and vary on a large scale between different geographic regions (Alloway, 1995). Elevated concentrations of metals in the soil environment mainly result from anthropogenic activities such as the extraction, smelting, and processing of metal-bearing ores, the distribution and use of metal-containing products, and the return of concentrated metals through the disposal of processing wastes and the discard of spent products (D'Amore, et al., 2005). These human activities have interfered with the biogeochemical cycles of metals that occur slowly in the natural environment.

Metal behavior

When metals are introduced into soils, a series of complex physical, chemical, and biological reactions (e.g., complexation/dissociation, oxidation/reduction, sorption/ desorption, and precipitation/dissolution) can take place. As a result, metals are distributed differently among fractions of the soil solid and solution phases (Roberts et al., 2005). Speciation is defined as the distribution of a metal over various chemical forms in the soil solid phases and soil solution (Peijnenburg et al., 2007). Metal can be bound in the soil solid phase by three types of reactions: (i) precipitation with anions such as hydroxides, carbonate, sulphate and phosphate, (ii) adsorption due to ion exchange, chemical bond formation, and complexation, and (iii) occlusion into the crystal structure of minerals (Levy et al., 1992). The soil solution phase may host the metal as free ion or complexed with inorganic (OH^-, Cl^-, HCO_3^-, SO_4^{2-}, NO_3^-, etc.) and organic ligands (dissolved organic carbon like humic and fulvic acids) (Almas et al., 2007; Roberts et al., 2005; Ure and Davidson, 1995). Solid-solution

partitioning of metals reflects the differences in chemical behavior and mobility of metals in different soils, to a large extent determining availability of metals to organisms and effects.

Metal effects

Concerns about the input of metals to soil are related to their ecotoxicological impact on organisms living in the soil. Next to effects on soil organisms, metal may be transferred via the food chain, resulting in health effects on animals and humans. Several metals (e.g., Cu, Ni, and Zn) are essential for living organisms because they play an important role in various biochemical and physiological processes, while some (e.g., Cd, Pb, and Hg) are highly toxic and have no known physiological function (nonessential) (Peijnenburg et al., 2007). There is an optimal level of essential metals for maximum benefit. Below this level symptoms of metal deficiency may occur whereas above this level the metal may become less beneficial and eventually toxic with increasing levels of availability (Hopkin, 1989). Toxic effects of metals on soil invertebrates (e.g., earthworms, enchytraeids, and nematodes) and microorganisms are reduced species diversity, abundance, and biomass (Bengtsson and Tranvik, 1989; Santorufo et al., 2012) and changes in microbial processes (e.g., glucose induced respiration and potential nitrification rate) (Oorts et al., 2006; Vig et al., 2003). Effects of metals on vascular plants can be reflected in the form of toxicity symptoms (reduced development and growth of shoots and roots) (Le et al., 2012), physiological symptoms (decreased nutrient contents in leaf tissues, elevated concentrations of total sugar and starch) (Prasad 1995), and biochemical symptoms (decreased enzymatic activity) (Das et al. 1997; De Vries et al., 2007). These effects could have serious consequences for soil biodiversity, and in turn soil functioning and ecosystem sustainability. Therefore, an understanding of the actual risks posed by metals in soil is needed.

Metal risk assessment

Ecological risk assessment for metals in soil is routinely conducted based on laboratory data from standardized ecotoxicity tests using selected terrestrial species (e.g., earthworms, Enchytraeids and Collembola) (Løkke and Van Gestel, 1998). These ecotoxicity tests focus on establishing quantitative concentration-effect relationships so that toxicity thresholds and therefore risk limits can be derived. This information is required in the modern regulatory system of the European Union - REACH (Registration, Evaluation and Authorization of Chemicals). REACH makes industry responsible for assessing and managing the risks posed by metals and other chemicals. Recently, there are also new moves toward establishing a Chinese REACH system: two guidelines concerning risk assessment under the order No.7 of the Ministry of Environmental Protection of China are pending (MEP, 2011). The original *Guideline for the Hazard Evaluation of New Chemical Substances* (HJ/T 154-2004) is revised into two separate documents, *the Guideline for Risk Assessment of Chemicals* and *the Guideline for Hazard Identification of New Chemical Substances*. The (draft) Guideline for Risk Assessment of Chemicals specifies the technical methods and procedures for the environmental and health risk assessment of chemical substances. The hazardous new chemical substances of priority environmental concern are screened out based on the outcome of risk assessment.

To date, soil quality criteria and risk assessment of metals are still predominantly based on total concentrations (Janssen et al., 2003). However, efforts to relate the total concentration of a metal to toxic effects have proven difficult (Reeder et al., 2006). Metal toxicity, even to the same organisms, can vary largely in different soils because of the impact of soil properties and metal bioavailability, which affects the relevant exposure concentration for organisms (Rooney et al., 2006; Criel et al., 2008; Santorufo et al., 2012). Improving the accuracy of the risk assessment and decision making requires an explicit consideration of bioavailability and other ecological knowledge.

1.2 Bioavailability

The concept of bioavailability was introduced to consider the fraction of a contaminant that will actually have an effect on organisms (Patrick et al., 1977; Mayer, 2002). However, bioavailability cannot be directly related to toxic effects as the latter follow from the amount of metals that react with the target sites of cells (Peijnenburg et al., 2007). As such, bioavailability can be considered a three-step approach, in which the available metal causes exposure (environmental availability), exposure leads to dynamic or passive uptake (environmental bioavailability), and subsequent effects result from reaction with a biological target (toxicological bioavailability) (Figure 1.1) (Dickson et al., 1994; Landrum et al., 1994; Peijnenburg et al., 1997).

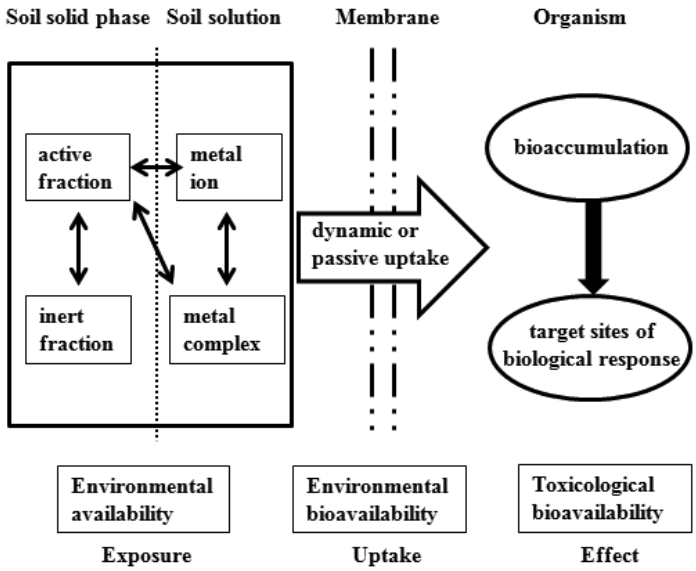

Figure 1.1 Schematic generalized depiction of the concept of bioavailability in soil systems. The active fraction is the metal that is available for exchange with the soil solution. The inert fraction is the metal that is occluded in mineral particles (clay, oxides) and organic matter. In soil solution, metals are present in dissolved species as metal ions and complexes. Metal interactions with various soil components affect actual exposure (environmental availability). Exposure leads to dynamic or passive uptake of

the metal across the membrane of orgamism (environmental bioavailability). The amount of accumlated metal that reacts with the target sites determines the subsequent effects (toxicoligical bioavailability).

Metals exist in different solid-phase and solution-phase forms that can vary greatly in terms of their bioavailability. Although the majority of metals are in the soil solid phase, metals in the solution phase pose the largest risks for soil dwelling organisms (Groenenberg, 2010). Metals in the soil solution are far more mobile and available than those in the solid phase (De Vries et al., 2005). Uptake by soil organisms via soil solution is supposed to be the most relevant pathway for metal exposure (Van Gestel and Koolhaas, 2004; Vijver et al., 2003). When a metal is supplied from the solid phase, it must first be transferred to solution before it can be taken up. Furthermore, not all dissolved metal species in the solution phase are readily available for uptake by organisms (Zhang et al., 2004). Therefore, the metal concentration and speciation in the soil solution are the major aspects that govern bioavailability. Extensive studies have reported that soil properties which control metal partitioning and speciation, such as pH, organic matter content, clay content, cation exchange capacity and the concentration of Fe- and Al-oxyhydroxides, have a significant influence on bioavailability (Janssen et al., 1997; Peijnenburg et al., 1999; Oorts et al., 2006; Smolders et al., 2009).

Besides physiochemical properties of the soil, biological processes may also play an important role in controlling bioavailability. For example, earthworms appear to affect metal bioavailability through stimulating soil microbial population, altering pH and DOC of the soil solution (Sizmur and Hodson, 2009). Plants can change metal bioavailability through root activities and related rhizosphere processes (rhizosphere acidification or root proliferation and secretion of organic acids). Mench and Martin (1991) reported that soil Cu, Cd, Ni, Zn, Fe, and Mn were dissolved by root exudates of corn.

A variety of chemical extraction methods are available for determining bioavailable fractions of metals in soil (Peijnenburg et al., 2007). These methods are usually operationally defined and are based on the correlation between water-soluble or exchangeable metal and biological effects (internal concentration or other endpoints) (Houba et al., 1996; Menzies et al., 2007). Chemical extraction methods are convenient, rapid, and cost-effective, and they thus provide data of significance for risk assessment practices. The limitations lie in that the extractant is often non-selective and non-exclusive, hindering the comparison between different soils. For different soil types, selection of a reasonable extractant and procedure is difficult. Several devices and methods have already been proposed to mimic bioavailability, such as the Donnan membrane technique (DMT) (Temminghoff et al., 2000), the device of diffusion gradients in thin films (DGT) (Zhang et al., 2001), physiologically based extraction tests (PBET) (Ruby et al., 1993), and simplified bioaccessibility extraction tests (SBET) (Medlin, 1997). Although the correlations between the results of these approaches and the effects of the specific metals seem reasonably good, these approaches do not provide any mechanistic information on bioavailability and toxicity. It is therefore difficult to develop an integrated approach to predict metal effects on organisms. To provide a mechanistic framework for site-specific risk assessment of metals, bioavailability models, which combine

chemical insights in metal speciation with biological insights regarding effects, have been developed. These models will be introduced below.

1.3 Effect modelling

Already for a long time researchers attempt to understand the exact mechanisms that induce differences in bioavailability and subsequent toxicity across water and soils. It has been recognized that metal bioavailability and toxicity are to a large extent controlled by the free ion activity in (soil) solution (Morel, 1983; Campbell, 1995; Batley et al., 2004; Van Gestel and Koolhaas, 2004). Based on these assumptions, mechanistically underpinned models such as the free ion activity model (Morel, 1983), the biotic ligand model (Di Toro et al., 2001) and the electrostatic model (Wang et al., 2008) were developed. Given the global desire of minimizing animal testing and reducing costs of regulatory testing of chemicals (Höfer et al., 2004), these modelling approaches are favorable for conducting ecological risk assessment of metals.

Free ion activity model

The bioavailability models for predicting metal toxicity were initially developed for the aquatic environment. In 1983, François Morel published a book "Principles of Aquatic Chemistry", which led to the development of the conceptual free ion activity model (Morel, 1983). The model describes how variations in the effect levels of metals can be related to their aqueous speciation and interactions with the organism (Morel, 1983; Paquin et al., 2002). It is assumed that among the various metal species, only the free metal ion can bind to the active sites (carrier, channel or toxic action sites) of the cell surface membrane, and subsequently be transported across the membrane to induce toxic effects (Morel, 1983; Campbell, 1995; Brown and Markich, 2000). Interactions between a metal ion and an organism generally involve three steps: (1) diffusion of the metal ion in the bulk solution to the surface of the cell membrane; (2) sorption or surface complexation of the metal ion at the active sites of the cell membrane; (3) uptake or transport of the metal ion across the cell membrane of the organism (Campbell, 1995). The key assumption which underpins the free ion activity model is that there is a rapid equilibrium between metal ions in the solution and those at the cell membrane (as follows):

$$\{M^{z+}\} + \{\text{-}R_{cell}\} \overset{k_1}{\leftrightarrow} \{M\text{-}R_{cell}\} \quad (1\text{-}1)$$

that is,

$$\{M\text{-}R_{cell}\} = k_1 \{M^{z+}\} \{\text{-}R_{cell}\} \quad (1\text{-}2)$$

where $\{M^{z+}\}$ is the free metal ion activity, $\{\text{-}R_{cell}\}$ is the concentration of free surface sites on the cell membrane, $\{M\text{-}R_{cell}\}$ is the activity of the surface complex, k_1 is the conditional stability constant. The biological response is assumed to be directly proportional to $\{M\text{-}R_{cell}\}$. The nature of the cell surface is assumed to remain unchanged during exposure (Hassler et al., 2004; Tessier and Turner, 1995). Therefore the biological response is determined by $\{M^{z+}\}$. Later studies found that even in exposure media with the same free metal ion activity, other factors such as hardness, DOC, and pH can affect metal toxicity (Allen and Hansen, 1996; Campbell, 1995). Based on these findings, the concept of the biotic ligand model has been

proposed and developed to consider both chemical speciation and ion competition in estimating metal toxicity (Di Toro et al., 2001; Paquin et al., 2000; Santore et al., 2002).

Biotic ligand model in the aquatic environment

The biotic ligand model (BLM) is a theoretical framework in which toxicity is related to the binding of metal ions to the sites of toxic action on an aquatic organism. For modelling purposes, the sites of toxic action are treated as a biotic ligand, that is, the biological counterpart of chemical ligand to which metals can bind. Toxic effects occur when the concentration of metal-biotic ligand complex reaches a certain critical level (Di Toro et al., 2001). The model also considers that other dissolved constituents that are present in the aquatic environment can influence the extent of metal binding to the biotic ligand. These constituents may either reduce the concentration of metal-biotic ligand complexes by competing with the metal for binding at the biotic ligand (e.g., Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) or by the formation of dissolved complexes with the metal (e.g., dissolved organic carbon, inorganic ligands) (Figure 1.2) which reduce the free metal ion activity. The principal feature in the BLM is the competition of the free metal ion with other cations for binding at the biotic ligand. This feature distinguishes the BLM from earlier concepts that considered only the free metal ion as the toxic species. The metal-organism interaction part of the biotic ligand model is based on the free ion activity model.

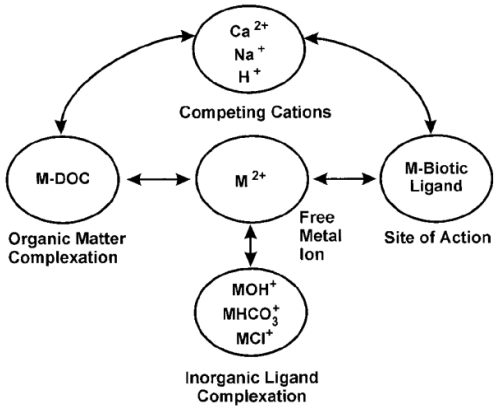

Figure 1.2 Schematic diagram of the framework of Biotic Ligand Model. For further information see the text and Di Toro et al. (2001).

For fish, the primary sites of toxic action for most metals is either known or suggested to be the Ca^{2+} or Na^{+} channel proteins in the gill surface (Pagenkopf, 1983; Di Toro et al., 2001). The amount of metal bound to the fish gill can be experimentally determined (Meyer et al., 1999). For the other species, the sites of toxic action are not readily accessible to direct measurement. Therefore, the term *biotic ligand* is adopted to facilitate the applicability of the BLM concept to other aquatic organisms. A detailed historical overview of the development of the aquatic biotic ligand model is given by Paquin et al. (2002), including an account of the primary sites of toxic action in aquatic organisms (i.e., the gill) and the associated

physiological processes involved in ion-regulation between the organism and the external environment. The most important adverse effects of metals on aquatic organisms have been demonstrated to involve the disturbance of the organisms' ability to regulate their internal ion balance. Three principal categories of physiological mechanisms of toxicity are distinguished: monovalent metals affecting Na^+ transport, divalent metals affecting Ca^{2+} metabolism, and metals that affect the organism centrally after passing the gill. Paquin et al. (2002) refer to various experimental studies demonstrating that metal accumulation at the site of toxic action is truly a function of metal complexation and competitive interactions, as conceptualized in the earlier models such as the gill surface interaction model (Pagenkopf, 1983) and the free ion activity model (Morel, 1983).

According to the assumption of the BLM, metal ions (M^{z+}) and other cations (H^+, K^+, Ca^{2+}, Na^+, and Mg^{2+}) can bind to the theoretical biotic ligand (BL) sites (De Schamphelaere and Janssen, 2002). The interaction between cations and BL is treated as a surface complexation reaction. At equilibrium, for example, the stability constant for M^{z+} binding to biotic ligands K_{MBL} (L/mol) can be expressed as a function of the concentrations of cation-biotic ligand complexes [MBL] (mol/L) and unoccupied biotic ligand sites [BL] (mol/L):

$$K_{\mathrm{MBL}} = \frac{[\mathrm{MBL}]}{\{\mathrm{M^{z+}}\}\times[\mathrm{BL}]} \tag{1-3}$$

where $\{M^{z+}\}$ is the free metal ion activity (mol/L).

Metal toxicity is assumed to be proportional to the fraction (f) of the total biotic ligand sites $[BL]_T$ occupied by the toxic metal. The f value depends on the binding affinity of M^{2+} to the BL and the presence and binding affinity of the competing cations (De Schamphelaere and Janssen, 2002):

$$f = \frac{[\mathrm{MBL}]}{[\mathrm{BL}]_{\mathrm{T}}} = \frac{K_{\mathrm{MBL}}\times\{\mathrm{M^{z+}}\}}{1+K_{\mathrm{MBL}}\times\{\mathrm{M^{z+}}\}+\sum K_{\mathrm{XBL}}\times\{\mathrm{X^{z+}}\}} \tag{1-4}$$

where $\{X^{Z+}\}$ is the activity of major cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+) in the solution, K_{XBL} is the binding constant of cations X^{Z+} binding to the BL.

The biological response is related to f using the logistic dose-response relationship:

$$R = \frac{R_0}{1+\left(\frac{f}{f_{50}}\right)^{\beta}} \tag{1-5}$$

where R is the survival rate, R_o is the control survival rate, f_{50} is the fraction of the total BL sites occupied by M^{2+} at which the survival rate is reduced by 50%, β is the slope parameter.

The value of f at the 50% effect level is assumed to be constant according to the BLM theory. Equation 2 then can be rewritten as:

$$\mathrm{EC50}\{\mathrm{M^{z+}}\} = \frac{f_{50}}{(1-f_{50})\times K_{\mathrm{MBL}}} \times \{1 + \sum K_{XBL} \times (\mathrm{X^{z+}})\} \tag{1-6}$$

where $EC50\{M^{z+}\}$ is the free metal ion activity inducing 50% effect. The BLM parameters required for predicting the effects are f_{50} and the binding constants of each cation. To date, the biotic ligand sites have been analyzed only for fish gills and f_{50} and binding constants have been experimentally determined for a number of metals (Playle et al., 1993). For the other species, these BLM parameters are generally obtained by fitting the model to observed measurements of toxic effects such as mortality. In other words, toxicity data are often used as the empirical basis for determining f_{50} and binding constants of metals to the biotic ligand

(De Schamphelaere and Janssen, 2002; Deleebeeck et al., 2009). The validity of the BLM can be established only if the critical concentration is the same over the entire range of environmental conditions that have been tested. A speciation model (e.g., WHAM) is needed for the necessary calculations. A major strength of the BLM is that it provides a mechanistic and operational framework for interpreting the effects of exposure water chemistry on metal bioavailability and toxicity. Many aquatic BLMs have been successfully developed and applied to predict metal toxicity to fish, algae, and water fleas in different exposure conditions (Alsop et al., 2000; De Schamphelaere and Janssen, 2004; Heijerick et al., 2002; Santore et al., 2002). The toxic effects estimated by these BLMs are generally within a factor of two of the observed toxic effects. Some exceptions are also reported especially in alkaline exposure conditions (De Schamphelaere and Janssen, 2002; Li et al., 2009a, Wang et al., 2009). Metal speciation changes substantially over the alkaline pH range and species other than the metal ion dominate in the solution (Wang et al., 2012). It has been suggested that the effect of pH on metal toxicity at a relatively high pH (>8) is a speciation effect rather than significant competition effects between protons and metal ions (De Schamphelaere and Janssen, 2002; Markich et al., 2003; Wang et al., 2010). In addition to free metal ions (e.g., Cu^{2+}), other metal forms (e.g., $CuOH^{+}$ and $CuHCO_3^{+}$) may also contribute to toxicity at pH-values exceeding 8. There is also the possibility of physiologically initiated modification of the structure of the membranes at high pH levels (Lavoie et al., 2012). Therefore, the applicability of BLM in high-pH media needs to be investigated further.

Biotic ligand model in the terrestrial environment

According to the BLM theory, toxicity occurs as a consequence of free metal ions reacting with the biotic ligand at the interface of solution and organism (Di Toro et al., 2001). It is assumed that the toxicity principles for fish and other aquatic species are also applicable to terrestrial species such as earthworms and plants. In case of these specific terrestrial organisms, the sites of toxic action are in direct contact with the external (soil) solution. This is an evolutionary phenomenon as general binding sites such as sodium and calcium transporters are inherent to every living cell (Niyogi and Wood, 2004). By considering the biotic ligand as a more general binding site, the principles underlying aquatic BLMs are likely to be valid also for terrestrial species. Attempts have been made to develop BLMs for soil invertebrates (earthworms and enchytraeids) (Steenbergen et al., 2005; Li et al., 2008; Lock et al., 2006) and plants (Lock et al., 2007; Li et al., 2009a) in solution or in solution-sand systems. These studies show that the application of the BLM to terrestrial organisms is theoretically and empirically feasible. One of the key limitations of these studies lies in the difficulty to relate the observed results to real world conditions. Increasing our understanding of the mechanisms behind metal uptake and toxicity to soil species is valuable, but the mechanistic information needs to be relevant with regard to the actual exposure of organisms in natural soils, for the terrestrial BLMs to be truly meaningful. The geochemistry of the hydroponic conditions bears little resemblance to that of real world soils, and identifying the challenges that are anticipated in soil validation of these systems would be appropriate.

A schematic diagram of the anticipated terrestrial BLM is presented in Figure 1.3, showing the interactions at the interface of the soil solid phase and the soil solution, and at the interface of soil solution and organism. Metals are exchanged between the soil matrix and

the solution by sorption to reactive solid phases, such as the soil organic matter (SOM), and the mineral (hydr)oxides of Fe, Al, and Mn, and clay. Competitive sorption of cations, protons, etc., to these solid phases also affects the distribution of the metal between the soil solid phase and solution. Free metal ions (M^{2+}) can form complexes with dissolved organic and inorganic ligands. A speciation model is needed to determine the metal speciation in solution, but such a model needs to account also for the partitioning of the metal between the soil solution and the soil solid phase. In addition to these soil geochemical processes that control metal speciation, the terrestrial BLM includes a toxicity model similar to that of the aquatic BLM.

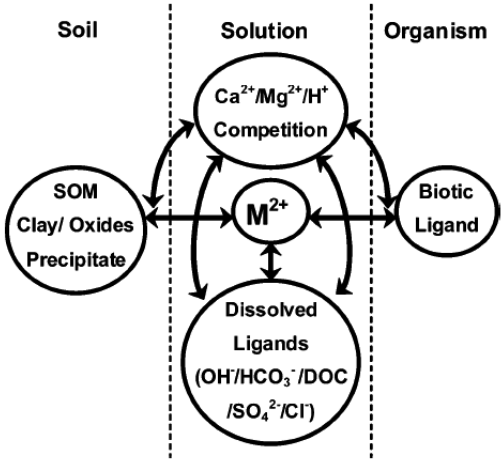

Figure 1.3 Schematic overview of the interactions considered in the terrestrial biotic ligand model (Thakali et al., 2006a).

The application of the BLM theory to soil toxicity data has rarely been done. This is because on the one hand there are not enough soil toxicity datasets suitable for such a purpose, the soil system is on the other hand much more complex than a solution system (route of exposure, environmental chemical behavior of metal) (Antunes et al., 2006; Plette et al., 1999). In soil, metal uptake by terrestrial organisms from the soil solution and the soil particles may in principle both be of substantial importance (Steenbergen et al., 2005). Like the aquatic BLM, the terrestrial BLM is based on the assumption of equilibrium partitioning. This assumption may not be valid when the surface complexation reactions between ions and biotic ligands are slow relative to the internal transport and the subsequent expression of the biological response. Besides, the environmental availability can be affected by dissolution kinetics of metal from the soil solid phase towards the soil solution phase. Equilibrium may also not be assumed when the metal transport into the organism is limited by diffusional control across the (static) boundary layer around the interfacial cells. As Thalaki et al. (2006a) argue, both kinetic control and diffusional limitations have been shown to occur, but are much more likely only under metal deficient conditions and thus not relevant to toxicity studies in general. Unlike the solution system it is difficult to univariately modify the parameters that affect metal toxicity in soil. Covariance exists among most of the

physicochemical properties of soil (Lofts et al., 2004; Wang et al., 2011a). For example, the amount of protons and major cations released to the soil porewater covaries with the amount of metal salt added in soil (Wang et al., 2011a). Parameterization of the BLM, therefore, faces a variety of challenges and uncertainties. Despite these difficulties, Thakali et al. (2006a, b) developed and applied the BLMs in acidic soils for describing Cu and Ni toxicity to plants (barley, tomato), soil invertebrates (earthworm, springtail) and microbial processes (nitrification potential, respiration). Free metal ions in the soil solution were assumed to be the dominant toxic species while cation competition was supposed to modify toxicity. Interactions between the metal and the soil solid phase, and between the metal and the solution phase were taken into account when calculating metal speciation in soil solution. In applying the BLM to the toxicity data, Thakali et al. found that it is unavoidable to empirically fix some of the BLM parameters (f_{50} and even one of the binding constants of the protective cations). Therefore, the estimated binding constants should not be regarded as conventional binding constants but rather as parameters that summarize the processes underlying the observed relation between organism responses and competing cations. Since the development of terrestrial BLMs for metals like Cu, Ni, and Cd is not readily feasible, it would be highly desirable to develop alternative methods, which facilitate the application of the BLM theory in soil, to assess metal toxicity to terrestrial organisms.

1.4 Mixture toxicity

In the real world, exposure to metal mixtures is a rule rather than an exception (Kortenkamp et al., 2009). However, current regulatory approaches focus almost exclusively on single metals and rarely require assessment of mixtures, which may have little real environmental relevance (Backhaus and Faust, 2012). The development of simple and efficient approaches for modelling mixture toxicity is necessary in the sense of meeting future regulatory demands and ensuring adequate risk assessment.

The foundations for mixture toxicity modelling have been laid by pharmacologists since 1920s (Loewe and Muischnek, 1926; Bliss, 1939). Conceptual models (see below) were developed by simply adding doses and responses to predict mixture effects based on the assumption that mixture components do not impact each other's toxicological action. However, the interactions of mixture components that may lead to decreased or increased bioavailability and toxicity were ignored. To date, our understanding of mixture toxicity is still based on those concepts, with addition as the basis for most models (Vijver et al., 2010). Only limited progress has been made to incorporate the interactions of mixture components in predicting mixture toxicity.

Conceptual models (Non-interaction)

Concentration Addition (CA) and Independent Action (IA) are the two main conceptual models that are available for calculating the toxic effects of mixtures, based on the toxicities of individual metals and their concentrations in the mixtures (Loewe and Muischnek, 1926; Bliss, 1939).

The CA concept was developed by Loewe and Muischnek (1926) to describe mixtures where the components have the same or a similar mode of toxic action (i.e., act on the same biological pathway and strictly the same molecular target):

$$\mathrm{CA} = \mathrm{TU_{mix}} = \sum_{i=0}^{n} \frac{c_i}{\mathrm{EC}x_i} \quad (1\text{-}7)$$

where n is the number of mixture components, c_i is the concentration of component i in the mixtures causing x_i% effect, ECx_i is the effective concentration of component i causing x_i% effect when applied singly. The term $\frac{c_i}{\mathrm{EC}x_i}$ is also defined as Toxic Unit (TU) which scales the relative toxicity of each component (Sprague, 1970). TU_{mix} is therefore a dimensionless quantity that equals the sum of the TUs of the individual components in the mixture. When the EC50 of the mixture equals 1 TU_{mix}, no interactions occur and CA holds. Values of TU_{mix} that are statistically significant below 1 indicate synergism, while values significantly exceeding 1 describe antagonism. CA deals with the issue that the relative toxicity of the metals that are present in the mixtures is the same as the relative toxicity of the metals present individually.

The concept of IA was first proposed by Bliss (1939) to describe mixtures where the components have different modes of action (i.e., act on different physiological systems):

$$\mathrm{IA} = E(c_{mix}) = 1 - \prod_{i=1}^{n}[1 - E(c_i)] \quad (1\text{-}8)$$

where $E(c_{mix})$ is the predicted effect (scaled in the range of 0-1) of mixtures, c_i is the concentration of component i, $E(c_i)$ is the effect of component i present singly at a concentration c_i. IA addresses the question whether the probability of response to one metal may be independent from the probability of response to another metal. In this model, the relative toxicity potency of metals is ignored, and the mixture effect is predicted from the joint probability of statistically independent events (Peijnenburg and Vijver, 2007).

With regard to the choice for a conceptual model, the basic idea is to use CA if the components are expected to act similarly and to use IA if the components are expected to act dissimilar (Junghans et al., 2006). However, identifying the modes of action for different chemicals is not always possible. In those cases, CA is suggested to be the more conservative choice in a risk assessment context as it estimates a higher response than IA and therefore represents the worst-case scenario for mixture exposure (Lock and Janssen, 2002; Backhaus and Faust, 2012).

Deviations from conceptual models (Interaction)

Although CA and IA are extensively used, it should be pointed out that both CA and IA approaches were put forward based on additivity, ignoring interactions between mixture components. This may explain why there are some scenarios in which the mixture effect cannot be fully described with either conceptual model. Multiple metals may interact with each other, which leads to more-than-additive (synergism) or less-than-additive (antagonism) effects. Besides, interactions between different mixture components may also depend on design of the toxicity test, such as the overall mixture concentrations and the relative proportion of component concentrations (Norwood et al., 2003; Jonker et al., 2005). In the present study, we therefore defined the following scenarios where deviations may occur:

(1) *Non-interaction*: the observed mixture effect is adequately explained by either CA or IA.

(2) *Absolute synergism or antagonism*: the observed effect of all combinations of a mixture is significantly more severe (synergism) or less severe (antagonism) than the mixture effect predicted by either CA or IA.

(3) *Dose ratio dependent deviation*: the deviation from the CA- or IA-predicted mixture effect depends on the relative proportion of mixture components. For example, in binary mixtures, antagonism can be observed when component A dominates toxicity, whereas synergism can be observed when component B dominates toxicity.

(4) *Dose level dependent deviation*: the deviation from the CA- or IA-predicted mixture effect depends on the overall mixture concentrations. For example, antagonism can be observed at low dose levels while synergism is observed at high dose levels.

In soil, mixture toxicity is complex to study because metals may interact at various levels (Calamari and Alabaster, 1980): (1) the exposure level, (2) the uptake level, (3) the target level, and (4) the internal pathway of detoxification. The first level deals with physicochemical interactions in the soil matrix, influencing sorption (partitioning of metals between soil solid phase and soil solution) and thereby the bioavailable fraction of metals. The second level involves physiological interactions during the uptake processes by the organisms, which affect toxicokinetics and subsequently the quantity available at the sites of action. The third and fourth levels describe interactions of metals at the receptors and target sites, at the intoxication processes, which affect toxicodynamics and hence the joint effect (Weltje, 1998; Conder and Lanno, 2000). Insight into these interaction levels and their relative importance is of great value for toxicity assessment of metal mixtures. This information will help to generalize study results between metal mixtures, as well as between different soil types and organisms.

Incorporation of bioavailability into mixture toxicity modelling

Another challenge in predicting mixture effects results from differences in the bioavailability and approaches used to define the bioavailable fraction among toxicological studies and subsequent ambiguity in interpreting mixture toxicity data (Peijnenburg and Vijver, 2007). For example, different conclusions (antagonistic, more antagonistic, additive) on mixture interactions were drawn when using different expressions of exposure (total soil concentration, $CaCl_2$-extractable concentration, internal concentration) (Bongers, 2007). The 0.01 M $CaCl_2$-extractable metal concentration or porewater concentration is often considered to be a suited estimate of the available metal pool for soil organisms (Peijnenburg et al., 2007). Therefore, all these expressions of exposure are considered in our research. Understanding the chemical interactions in soil could help in the interpretation of different outcomes of studies working with soils of different types.

Biotic ligand models for predicting toxicity of individual metals have been reported for more than a decade (Di Toro et al., 2001; Paquin et al., 2002). Only recently has the BLM been extended to consider mixture scenarios (Jho et al., 2011; Le et al., 2013). The basic assumption underlying the BLM (ion competition) potentially allows incorporating mixture interactions into the assessment of mixture toxicity. If two metals compete for binding to the same biotic ligand, it is possible to estimate the total amount of metal bound to that BL and,

hence, to predict metal toxicity using the BLM in a CA model. Alternatively, if competitive binding does not occur, then the BLM may provide more reliable estimates of bioavailability of individual metals, which can then be incorporated into a more accurate IA model.

1.5 Species-specific responses

Metal accumulation and effects are not only driven by abiotic factors like chemical speciation and cation competition. Organisms themselves have developed effective strategies to cope with metal exposure. They have the ability to excrete or eliminate the metal to control metal accumulation and maintain homeostasis over a certain level of exposure (Chapman et al., 1996; Wood, 2001). They are also able to minimize toxic effects of reactive forms of metals in their body by sequestration, detoxification, and storage (Vijver et al, 2004). These physiological processes of organisms have not been accounted for when assessing the risks posed by metals in the environment. Studies have shown that metal accumulation and excretion rates are species dependent. Rapid zinc uptake and elimination was found in *Eisenia fetida* (Spurgeon and Hopkin, 1999), while slow uptake was found in *Lumbricus rubellus* (Mariño and Morgan, 1999). Janssen et al. (1991) studied the accumulation and elimination kinetics of cadmium in four arthropod species and observed large differences between species, which could mainly be attributed to species-specific differences in accumulation strategies. Variations in sensitivity to a given metal have been reported widely between different species. For example, Spurgeon et al. (2000) found that the earthworms *L. rubellus* and *Aporrectodea caliginosa* were more sensitive to Zn than *L. terrestris* and *E. fetida*. Langdon et al. (2005) reported that the sensitivity of three earthworm species to Pb followed the decreasing order: *L. rubellus* > *A. caliginosa* > *E. fetida*. This can be a result of species-specific differences in physiological characteristics that determine detoxification and elimination strategies (Dallinger, 1993). In addition, the activity of calcium glands in earthworms may partially account for the differences in sensitivity as calcium is involved in the sequestration and elimination of many metals. It has been suggested that the more tolerant species *E. fetida* and *L. terrestris* have a higher calcium gland secretion activity than the relatively sensitive species (Morgan and Morgan, 1991; Spurgeon and Hopkin, 1996). Other species characteristics (e.g., time to maturity, habitat, food choice, immune-competent cells, etc.) may also affect metal accumulation and toxicity (Edwards and Bohlen, 1996; Plytycz et al., 2011a). More research efforts are needed on this aspect to establish solid links between species-specific responses and the relevant traits (i.e., specific characteristics of species) and processes. The exploration of such an approach, that is a traits-based approach (Baird et al, 2008; Rubach et al, 2012), might assist in explaining why one species is more sensitive to metals than another species and allows for extrapolating the results of metal accumulation and toxicity over species.

1.6 Earthworms

Earthworms constitute a large proportion of the total biomass of soil animals and play a vital part in the natural soil ecosystem. They are key species within the soil food web, which is a complex community of soil-dwelling organisms that influence the processes of soil

formation (Peijnenburg and Vijver, 2009). They are also valued for their contribution to ecosystem services (i.e., provisioning, regulating, supporting and cultural services) through their action on soil processes (Blouin et al., 2013). Earthworms are commonly considered as soil ecosystem engineers because they benefit the soil ecosystem in a number of ways: mixing soil layers, recycling organic matter, increasing nutrient availability, improving soil aeration, and enhancing microbial activity, et cetera (Edwards and Arancon, 2004; Blouin et al., 2013). As earthworms play a unique role in sustaining the essential functions of soil, detrimental effects of metals on earthworms may indirectly harm soil health and subsequently the whole ecosystem.

Different species of earthworm have different habitats, behaviors, and life histories and occupy different niches within the soil ecosystem (Domınguez, 2004). Earthworm species are generally divided into three different functional groups based on their ecological strategies (Bouché, 1977): epigeic, endogeics, and anecic (Figure 1.4). Epigeic species live in the soil surface layer and feed on litter and organic rich materials. Endogeic species live in the mineral layer of the soil and feed on soil and associated organic matter. Anecic species live in deep vertical burrows but feed on litter at the soil surface (Domınguez, 2004). The Figure 1.4 illustrates where the earthworms live (their habitats), indicating that these earthworm species occupy different ecological niches.

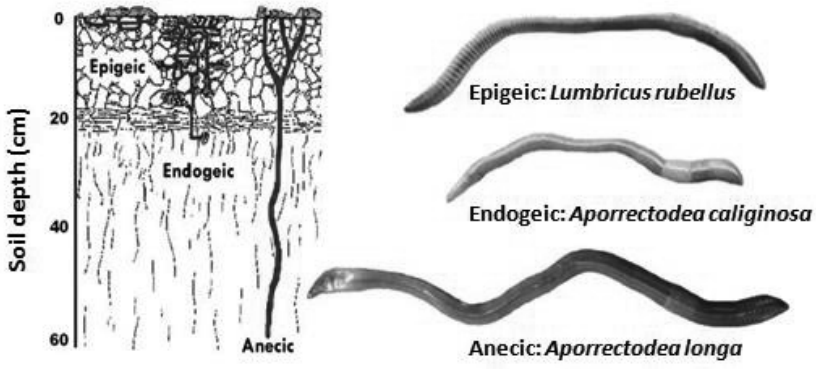

Figure 1.4 Earthworm functional groups and representative species of each group. Figure adapted from Fraser and Boag (1998) (slightly modified).

Earthworms have been considered as feasible biological indicators for metal pollution in soils due to their ecological importance, high sensitivity and great abundance (Dittbrenner et al., 2011; Maleri et al., 2007). A series of test guidelines and protocols have been developed by the Organisation for Economic Co-operation and Development, i.e., "Earthworm Acute Toxicity Tests, Guidelines for the Testing of Chemicals, No. 207" (OECD, 1984), and "Earthworm Reproduction Test (*Eisenia fetida*/*Eisenia andrei*), Guidelines for the Testing of Chemicals, No. 222" (OECD, 2004), and by the International Organization for Standardization, i.e., "Soil quality -- Effects of Pollutants on Earthworms -- Part 1: Determination of acute toxicity to *Eisenia fetida*/*Eisenia andrei* ISO 11268-1: 2012; Part 2:

Determination of Effects on Reproduction of *Eisenia fetida*/*Eisenia andrei*, ISO 11268-2: 2012" (ISO, 2012). These guidelines are designed to estimate critical effect levels from concentration-response relationships for survival and/or reproduction of mature earthworms.

Earthworms are ideal organisms for assessing the toxicity of metals to soil-dwelling species, because they are in intimate contact with the soil porewater and the soil solid phase. Thus, they are exposed in a manner representative of many soil species, including bacteria, plants, and other soft-bodied invertebrates. Earthworms have a water-permeable surface epithelium. Although direct uptake via the gut wall (food) cannot be completely ruled out, many findings have suggested that metal uptake by earthworm takes place predominantly via the porewater or via an uptake route that is related to porewater uptake (i.e., the porewater hypothesis) (Saxe et al, 2001; Vijver et al., 2003; Jager et al., 2003; Van Gestel and Koolhaas, 2004). Vijver et al (2003) investigated a method (oral sealing using glue) to distinguish different uptake routes of metals in earthworms and found that the dermal route is the main uptake route. Janssen et al (1997) found that the same soil properties affecting metal partitioning between the soil solid phase and soil porewater were also the dominant soil properties affecting metal accumulation in earthworms. In our study, the porewater hypothesis was adopted. Metal in soil porewater is assumed to dominate toxic effects, which connects the soil solid phase and the organism.

Four earthworm species (*Eisenia fetida, Lumbricus rubellus, Aporrectodea caliginosa and Aporrectodea longa*) were used in the present study. The epigeic *E. fetida* is an artificially cultured species that inhabits only organic matter-rich locations. They are rarely found in natural soil. *L. rubellus, A. caliginosa and A. longa* are the representatives of epigeic, endogeic, and anecic earthworm species living in natural soil, respectively (Figure 1.4) (Spurgeon et al., 2000). The differences in ecological strategies and physiological characteristics between species may strongly affect effective exposure and toxicity (Morgen and Morgen, 1999). Taxonomy is not an inherently informative indicator for prospective risk assessment of metals, as two species of the same genus may show large differences in sensitivity. However, the phylogenetically related aggregations of certain species traits may provide a clue for species-specific accumulation and toxicity (Rubach et al. 2010). It has been suggested that uptake processes are physiologically driven and affected by species specific parameters (traits) such as morphology, soil habitat, feeding strategy and preferences, and life history (Peijnenburg et al., 2012). Species may possess different trait combinations to cope with a particular disturbance (De Lange et al. 2013). Incorporating traits-based approaches in metal toxicity assessment is supposed to give more explanatory power and allows for extrapolation of results of metal accumulation and toxicity between different earthworm species.

1.7 This thesis

Objective

As reviewed above, uncertainties or deviations still exist when predicting the adverse effects of metals and metal mixtures on soil organisms in different exposure scenarios. This thesis has the overall aim to improve ecological risk assessment for metals through better

understanding bioavailability and developing appropriate bioavailability models for soil organisms. Therefore, the specific goals of this thesis were:

1. To develop bioavailability models to facilitate the application of BLM theory in soils;
2. To model mixture toxicity by taking into account the interactions of mixture components at different toxicological levels;
3. To extrapolate the study results to other studies reported in the literature.

To accomplish these goals, the following research questions were addressed:

[1] Which cations (H^+, Ca^{2+}, Mg^{2+}, K^+ and Na^+) exert significant effects on metal toxicity and how could these toxicity-modifying factors be incorporated into terrestrial toxicity models developed on the basis of the BLM theory? (Chapters II and III)

[2] Are the toxicity-modifying factors the same for different earthworm species (*Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*) and for different metals (Cu, Cd, and Ni)? (Chapters II and III)

[3] Are metal (Cu, Cd, Ni, and Zn) accumulation pattern and sensitivity of earthworms species-specific? Can species-specific traits of earthworms provide a clue for predicting metal accumulation and toxicity? (Chapter IV)

[4] Where do the interactions of mixture components (Cd and Zn) possibly occur and how do they impact the observed toxicity? (Chapter V)

Biotic ligand models are increasingly being developed and applied to relate toxic effects of metals on aquatic organisms to activities of the free metal ion and competing ions. We have adopted the recent hypothesis that this modelling theory is also applicable to organisms in the soil environment. It is expected that free metal ions in soil porewater are mainly responsible for toxicity while the base cations can mitigate toxicity through competition for binding to the biotic ligand sites. Trait-based approaches are used to assist in explaining differences in metal accumulation and toxicity between earthworm species living in different habitats, with ultimately the idea to enable cross-species extrapolation of accumulation and toxicity data. Since mixture exposure represents realistic field scenarios and since mixture interactions at different toxicological levels may impact the actual risks, attempts are made to determine where mixture interactions could occur and to quantify the toxicity deviations from simple concentration addition, ultimately ensuring adequate risk assessment.

Thesis outline

This Chapter provides an overview of the basic principles of speciation, bioavailability and effect modelling of heavy metals in the terrestrial environment, particularly with regard to the development of biotic ligand models and mixture toxicity models. The main issues were highlighted and ways to tackle the associated problems were discussed.

The research questions are answered in the following chapters:

Chapter II: A Freundlich-type model, rather than the biotic ligand model, was proposed to link Cu toxicity in different earthworm species (*L. rubellus*, *A. longa* and *E. fetida*) to free Cu ion activities and activities of possibly protective cations in soil porewater. This model

complies with the basic assumptions of the biotic ligand model but requires fewer parameters than the biotic ligand model, thus facilitating the application of biotic ligand model principles in soil exposure systems. The possibility of extrapolating the study results to other studies reported in literature was also explored.

Chapter III: When applying the BLM to the soil system, parameterization of the BLM faces a variety of challenges and uncertainties. Based on empirical studies and BLM theory, here we proposed an alternative method, the free ion approach, to predicting Cd and Ni toxicity to earthworms (*L. rubellus* and *A. longa*). Previously, the applicability of the free ion approach for describing Cu toxicity has been proven. However, the toxicity-modifying factors (H^+, Ca^{2+}, Mg^{2+}, Na^+, and K^+) are shown to be different for other metals. Results obtained from our study strongly suggest that metal toxicity to earthworms needs to be evaluated on a metal-specific basis.

Chapter IV: It is often stated that there is not enough ecology in ecotoxicology and that this lack can have unfortunate consequences for environmental risk assessment. Here we examined the differences in metals (Cu, Cd, Ni, and Zn) accumulation and toxicity in three earthworm species (*L. rubellus*, *A. longa* and *E. fetida*), with a special focus on the impact of earthworm traits. In this study, the ecophysiological differences between earthworms were identified and used to assist in explaining metal accumulation patterns and sensitivity. These species-specific traits of earthworms are expected to provide a clue for extrapolation across species.

Chapter V: The toxic unit method was used to quantify the mixture (Cd and Zn) toxicity in one earthworm species (*A. caliginosa*). Deviations caused by mixture interactions were assessed using the MIXTOX model. Interactions associated with different expressions of exposure (total, $CaCl_2$-extractable, and porewater concentrations) were compared. In soil, mixture toxicity is complex to study because interactions of metals can occur at various levels. By separating the interactions at the exposure level from the uptake level and the target level, it was determined where the interactions possibly occur and their influence on the toxicity pattern of binary metal mixtures was subsequently assessed.

Chapter VI: The results reported in Chapter II-V were summarized and synthesized. Based on the synthesis, this chapter further provides recommendations for the potential application of the developed models and approaches, and discusses briefly the areas of future research.

Chapter II

Predicting Copper Toxicity To Different Earthworm Species Using A Multicomponent Freundlich Model

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Abstract

This study aimed to develop bioavailability models for predicting Cu toxicity to earthworms (*Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*) in a range of soils of varying properties. A multicomponent Freundlich model, complying with the basic assumption of the biotic ligands model, was used to relate Cu toxicity to the free Cu^{2+} activity and possible protective cations in soil porewater. Median lethal concentrations (LC50s) of Cu based on the total Cu concentration varied in each species from soil to soil, reaching differences of approximately a factor 9 in *L. rubellus*, 49 in *A. longa* and 45 in *E. fetida*. The relative sensitivity of the earthworms to Cu in different soils followed the same order: *L. rubellus* > *A. longa* > *E. fetida*. Only pH not other cations (K^+, Ca^{2+}, Na^+, and Mg^{2+}) were found to exert significant protective effects against Cu toxicity to earthworms. The Freundlich-type model in which the protective effects of pH were included, explained 84%, 94%, and 96% of variations in LC50s of Cu (expressed as free ion activity) for *L. rubellus*, *A. longa*, and *E. fetida*, respectively. Predicted LC50s never differed by a factor of more than 2 from the observed LC50s. External validation of the model showed a similar level of precision, even though toxicity data for other soil organisms and for different endpoints were used. The findings of the present study showed the possibility of extrapolating the developed toxicity models for one earthworm species to another species. Moreover, the Freundlich-type model in which the free Cu^{2+} activity and pH in soil porewater are considered can even be used to predict toxicity for other soil invertebrates and plants.

2.1 Introduction

Soil contamination with metals poses a serious threat to soil functions and sustainability of ecosystems (De Boer et al., 2011). A large amount of Cu discharge comes with the widespread use of this metal, for example, in mining industry as a floatation reagent and in agriculture as a fungicide or fertilizer (Gimeno-García et al., 1996). This strengthens the need to develop appropriate quality criteria and predictive models to evaluate to what extent and what magnitude Cu poses risks to soil organisms.

Numerous studies have shown that uptake and effects of metal depend on species, soil type and metal bioavailability (Nahmani et al., 2007; Peijnenburg et al., 2007; Van Gestel et al., 2004). To induce potential effects, metal should be bioavailable for being taken up by soil organisms (Peijnenburg et al., 2007). The porewater hypothesis proposes that exposure takes place via the porewater or that uptake of metal is mediated by a porewater related route (Van Gestel and Koolhaas, 2004; Vijver et al., 2003). The free metal ion in soil porewater is supposed to be the potential toxic species that is actually taken up by soil organisms (Morel, 1983; Peijnenburg et al., 1999). This forms the theoretical basis of using the free metal ion to predict uptake and toxicity and leads to the free ion activity model (Morel, 1983). The development of the biotic ligand model (BLM) is an extension of the free ion activity model (Campbell, 1995; De Schamphelaere and Janssen, 2002). It considers metal speciation and competition with other cations (e.g., H^+, Na^+, K^+, Ca^{2+}, Mg^{2+}). Toxicity is assumed to be proportional to the fraction of the total biotic ligand sites (transport sites or physiologically active sites) occupied by the toxic metal (De Schamphelaere and Janssen, 2002; Niyogi and Wood, 2004). Initially BLMs were proposed as a tool to quantitatively predict metal toxicity for aquatic organisms. However, the principles underlying aquatic BLMs are likely to be valid also for terrestrial species (Plette et al., 1999), especially when exposure is predominantly via the dermal route and soil organisms (such as earthworms) are in close contact with the soil porewater (Steenbergen et al., 2005; Vijver et al., 2003).

Van Gestel and Koolhaas (2004) studied the toxicity of Cd to the springtail *Folsomia candida* and found that besides the free Cd ion activity, pH in soil porewater or in water extractable fraction influenced toxicity while calcium had a mitigating effect on toxicity. This suggested that the BLM approach may be applicable for soil-dwelling organisms. A Cu-BLM was developed for the earthworm *Aporrectodea caliginosa*, exposed in solution-sand system (Steenbergen et al., 2005). Increasing H^+ and Na^+ activities mitigated Cu toxicity while increasing Ca^{2+} and Mg^{2+} activities had inconsistent effects. The final Cu-BLM developed incorporated the effects of H^+ and Na^+. The verification of the BLM in artificially contaminated soil showed that, in some cases, predictions were not within the 95% confidence interval. To predict Co toxicity to the potworm *Enchytraeus albidus*, a Co-BLM, which considered the effects of H^+, Ca^{2+}, and Mg^{2+}, was developed using the exposure system with solution and quartz sand (Lock et al., 2006). The developed model was validated in a standard artificial soil and a standardized field soil. LC50s of Co were accurately predicted, that is, by an error of less than a factor of 2. Based on toxicity tests using a range of European soils of varying properties (Criel et al., 2008; Oorts et al., 2006; Rooney et al., 2006), Thakali and co-workers have developed terrestrial BLMs for predicting Cu and Ni toxicity to soil invertebrates, plants, and microbial processes (Thakali et al., 2006a; 2006b).

Some cases suggested the usefulness of the BLM to predict metal toxicity in soil, while others showed exceptions (Lock et al., 2006; Steenbergen et al., 2005; Thakali et al., 2006a; 2006b). This raises the question of whether the BLM concept is too simplified for complex soil processes.

When developing BLMs for terrestrial organisms, solution system, instead of the real soil, is often used in order to simplify the complex soil processes (Lock et al., 2006; Steenbergen et al., 2005). There may be two problems hindering the development of BLMs directly from soil system and the interpretation of data. Unlike the solution system, it is difficult to univariately modify the parameters that affect metal toxicity in soil. Another difficulty is that the intercorrelation among parameters in soil culture, for example, the amount of H^+, Ca^{2+}, and Mg^{2+} released to the soil porewater covaried with the amount of metal added in soil (Wang et al., 2011a). Even so, the soil exposure system was chosen in the present study considering the environmental reality. A multicomponent Freundlich model (Plette et al., 1999), rather than the BLM, was proposed to link Cu toxicity in different species of earthworms to free Cu^{2+} and possible protective cations in soil porewater. This model complies with the basic assumptions of BLM but requires fewer parameters than the BLM (Mertens et al., 2007; Ore et al., 2010), facilitating the application of BLM principles in soil exposure system.

The main objectives of the present study were to examine whether the multicomponent Freundlich model, which complies with the BLM concept and incorporates cations competition, can effectively predict Cu toxicity across different earthworm species (*Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*), and to explore the possibility of extrapolating the study results to other studies reported in literature.

2.2 Materials and methods

Soil spiking

Soils of varying properties were collected from six different agricultural sites (Valkenswaard, Boxtel, Woerden, Drimmelen, Vlaardingen, and Mook) in The Netherlands. The soil was air-dried, homogenized and passed through a 2 mm sieve before use. These soils were spiked with Cu acetate (Acros Chemicals; purity 98%) to achieve designed levels of concentrations (from 12.5 to 4000 mg/kg depending on soils; for details see Figure S2.1 in the Supporting Information (SI)) including a control. After spiking, the soils were subjected to alternation of wetting and drying at 35 °C in a temperature cabinet for two months to eliminate acetate by mineralization. A previous study showed that the net results of hydrolysis of released Cu^{2+} and acetate mineralization exerts unnoticeable effects on soil pH (Qiu et al., 2011).

Organisms

Earthworm species used in the toxicity tests were *Eisenia fetida*, *Lumbricus rubellus*, and *Aporrectodea longa*. These species were selected because they represent a range of earthworm ecotypes. *E. fetida* (OECD recommended species) is not a species inhabiting soils from nature but they are cultured and released into the natural soils. They inhabit only organic matter-rich locations, such as animal manure or compost heaps. *L. rubellus* is epigeic,

living in the uppermost 5 cm of soil and litter layers (Spurgeon et al., 2000). *A. longa* is anecic and lives in deep, permanent burrows. *E. fetida* was purchased from the Earthworm Cultivation Farm (Regenwormen, NL). Mature earthworms *L. rubellus* and *A. longa* were collected from an unexploited grassland soil located in Leiden, The Netherlands. Prior to the experiments the earthworms were acclimated in the unspiked soils for at least one week in the laboratory at 15 ± 2 °C.

Toxicity tests

Adult earthworms with a clearly developed clitellum were used. The earthworms with weight ranging from for 600 to 800 mg for *E. fetida*, 800 to 1000 mg for *A. longa*, and 700 to 900 mg for *L. rubellus* were selected for testing. Exposures were conducted in a climate room at 15 °C with an 8h-light: 16 h-dark cycle. Four earthworms were put into a plastic jar containing 500 g soil of different treatments. Each treatment was performed in triplicate. All soils were maintained at 80% maximum water holding capacity. Deionized water was added every week to compensate for water evaporation. The earthworms were fed with organic-rich food (5 g of cow manure per jar per week) during the experiment. Soil was aerated and mortality was checked every week and the dead worms were removed. After 28 days of exposure, the surviving number and fresh body weights of earthworms in each treatment were recorded (OECD, 2004). In all unspiked soils, mortality of the earthworms was less than 10% and no significant weight loss ($p > 0.05$) was observed.

Chemical analysis

Total Cu concentrations in soil samples were determined after digestion with *aqua regia*. Soil porewater was collected by means of suction over a 0.45 μm acetate filter of soil samples stored for one week at 15 °C at 100% of their maximum water holding capacity. Soil pH in 0.01 M $CaCl_2$ extracts and in porewater samples was measured using a pH meter (691, Metrohm AG) at the end of the test. Some soil samples were taken before, during, and after the tests. No noteworthy differences in soil pH were observed among different sampling periods. At the end of the test, soil texture, organic matter content (OM), and cation exchange capacity (CEC) were determined following the methods described by Pansu and Gautheyrou (2006). Dissolved organic matter in soil porewater was determined by a TOC/DOC analyzer (TOC-VCSH, Shimadzu). A copper ion-selective electrode coupled with a voltmeter with 0.1 mV resolution (Cole-Palmer, Copper Electrode) were used to measure the free Cu^{2+} activity (denoted $\{Cu^{2+}\}$) in the soil porewater. Standard stock solutions of $Cu(NO_3)_2$ of known activities at pH 2 were used to generate calibration curves for measuring $\{Cu^{2+}\}$ according to the Nernst equation. Concentrations of dissolved Cu, K, Ca, Na, and, Mg were measured by flame atomic absorption spectrophotometry (AAS, Perkin-Elmer 1100B). The detection limits of flame AAS for Cu, K, Ca, Na, and, Mg were 1.5 μg/L, 3 μg/L, 1.5 μg/L, 0.3 μg/L, and 0.15 μg/L, respectively. Standard reference material for soil (ISE 989) were used for each of 30 samples for the purpose of analytical quality control. Measured concentrations by flame AAS generally were in good agreement (±10%) with certified reference values.

Data analysis

Median lethal concentrations (LC50s) of Cu for each earthworm species in each soil were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). The Windermere Humic-Aqueous Model (WHAM VI) (Tipping, 1998) was used to calculate the activities of Cu^{2+} and other cations in soil porewater. Input data include porewater pH, colloidal fulvic acid (FA), dissolved Cu, major cation and anion concentrations (Ca, Mg, Na, K, Cl^- and SO_4^{2-}). It was assumed that 65% of DOC was active (available for metal binding) as the colloidal FA, while the remaining 35% was inert (Tipping et al., 2003). Presence of dissolved Cl^- and SO_4^{2-} in a molar ratio of 6:1 was assumed to maintain electroneutrality (Thakali et al., 2006a). For all the calculations in the present study, measured concentrations were used unless otherwise stated.

Modelling theory

A multicomponent Freundlich model was used to describe the Cu binding to the biotic ligand as it has conceptual and practical advantages over the BLM (Mertens et al., 2007; Ore et al., 2010; Plette et al., 1999). The Freundlich type model considers site heterogeneity, while in the BLM biotic ligands are considered to be chemically homogeneous with a single ligand-binding constant. Furthermore, it is flexible to describe, for example, that the effect of cations on Cu toxicity is pH dependent. By incorporating the competitive effect of protons and protective ions on Cu sorption to the biotic ligands, this model reads:

$$[\mathrm{CuBL}] = k\,\{\mathrm{Cu}^{2+}\}^{n\mathrm{Cu}}\,\{\mathrm{H}^{+}\}^{n\mathrm{H}}\prod\{\mathrm{C}_i^{z+}\}^{n\mathrm{Ci}} \quad (2\text{-}1)$$

where [CuBL] is the amount of Cu assumed to be bound to the biotic ligands, k, n_{Cu}, n_H, and n_{Ci} are the Freundlich parameters, and $\{Cu^{2+}\}$, $\{H^+\}$, and $\{C_i^{z+}\}$ (i.e., $\{Na^+\}$, $\{Ca^{2+}\}$, $\{Mg^{2+}\}$, and $\{K^+\}$) are ion activities (mol/L) in soil porewater. The free Cu^{2+} activity inducing 50% mortality ($LC50\{Cu^{2+}\}$) of earthworms in different soils is assumed to be associated with a given constant [CuBL] according to the BLM theory (De Schamphelaere and Janssen, 2002). Therefore, equation 2-1 can be transformed as follows:

$$\log LC50\{\mathrm{Cu}^{2+}\} = \alpha\,\mathrm{pH} - \sum \beta_i \log\{\mathrm{C}_i^{z+}\} + \gamma \quad (2\text{-}2)$$

where the coefficients α ($= n_H/n_{Cu}$), β_i ($= n_{Ci}/n_{Cu}$), and γ are constants. Stepwise multiple linear regression analysis was carried out using SPSS 16.0 (IBM) to decide which toxicity-modifying factors (H^+, Na^+, Ca^{2+}, Mg^{2+}, and K^+) need to be included in the model. This model was further applied to predict Cu toxicity to a range of other soil organisms with different endpoints using the underlying data in the literature (Criel et al., 2008; Oorts et al., 2006; Rooney et al., 2006; Thakali et al., 2006a; 2006b).

The general practice in applying the toxicity model to the data is to calculate individual toxic endpoints (LC50 or EC50) for each species in each soil (i.e., point estimates of toxicity), as the best test of a model's predictive ability is how well it predicts LC50 or EC50 (Thakali et al., 2006a). However, it may also be possible to extend the multicomponent Freundlich model (equation 2-2) to consider the entire dose−response curve. Although γ is constant at a given effect level, it will vary according to the effect level being described. The coefficient α and β_i, describing the effects of cations on Cu toxicity, are assumed to be independent of the effect level (Lofts et al., 2004). Generalizing to any effect level, the model reads

$$\log\{\mathrm{Cu}^{2+}\}_{\mathrm{EFFECT}} = \alpha\,\mathrm{pH} - \sum\beta_i \log\{\mathrm{C}_i^{z+}\} + \gamma_{\mathrm{EFFECT}} \quad (2\text{-}3)$$

or:

$$\gamma_{EFFECT} = \log\{Cu^{2+}\}_{EFFECT} - \alpha\, pH + \sum \beta_i \log\{C_i^{z+}\} \quad (2\text{-}4)$$

here, γ_{EFFECT} can be interpreted as the effect dose that incorporates not only the $\{Cu^{2+}\}$, but also the terms describing the effects of bioavailability, and differences in inherent sensitivity of organisms to Cu. $\{Cu^{2+}\}_{EFFECT}$ is the corresponding value of $\{Cu^{2+}\}$ at any given effect level.

The entire data set for each earthworm species were fitted with a logistic dose−response curve (equation 2-5) (Haanstra et al., 1985) using total Cu concentration, $\{Cu^{2+}\}$, and γ_{EFFECT} as dose, respectively.

$$R = \frac{R_0}{1 + (\frac{x}{x_{50}})^{\beta}} \quad (2\text{-}5)$$

where R = response, R_0= control response, x = total Cu concentration, $\{Cu^{2+}\}$, and γ_{EFFECT}, x_{50} = concentration (dose) at the 50% effect level, and β = shape parameter. R was plotted against x to fit the parameters x_{50} and β. The model parameters were estimated by minimizing the RMSE (root-mean-square error) using the SOLVER program in Microsoft Excel 2010.

2.3 Results and discussion

Soil and porewater properties

The most important properties of the unspiked soils are presented in Table 2.1. These soils represented a range of soil types and varied in soil pH, OM, and CEC, etc. The selected properties of soil porewater are listed in Table S2.1 in the SI. Soil porewater pH prior to spiking ranged from 5.0 ± 0.2 to 8.0 ± 0.3. The addition of different amounts of Cu (0-4000 mg/kg) only induced marginal effects (usually < 0.3 units) on the soil porewater pH (Figure S2.1 in the SI). It has been reported that the effects of Cu spiking on soil properties can be reduced to a minimum by using Cu acetate instead of other Cu salts (e.g., $CuCl_2$, $Cu(NO_3)_2$, and $CuSO_4$) (Qiu et al., 2011). Dissolved Cu concentrations in soil porewater increased with increasing total Cu concentrations. Significant linear relationships between porewater Cu and total Cu were found for all soils ($p < 0.0001$) (Table S2.2 in the SI). The significance of the regression equations was not improved by the inclusion of porewater pH or DOC as explanatory variables. The relationship between calculated and measured pCu ($-\log\{Cu^{2+}\}$) in the porewater of all soils is shown in Figure S2.2 in the SI. The free Cu^{2+} activities spanned almost 6 orders of magnitude. A significant correlation between calculated and measured pCu was observed ($R^2 = 0.85$, $n = 75$, $p < 0.0001$, $F = 369.6$). WHAM VI provided robust predictions of free Cu^{2+} activities even for the alkaline soils. According to a soil solid-solution partition model for metals (Lofts et al., 2004), pCu in the porewater of all soils conformed to a equation: pCu = 0.714pH − 0.211log(total Cu) + 3.565log(OM) + 5.203, (R^2 = 0.954, $p < 0.001$; total Cu in mg/kg, organic matter content (OM) in %), as obtained by multivariate linear regression. This suggested that metal partitioning and speciation was to a large extent determined by the soil pH and organic matter content.

Table 2.1 Selected soil properties of the unspiked soils. All values are given as means of three replicates.

	Valkenswaard	Boxtel	Woerden	Drimmelen	Vlaardingen	Mook
pH[a]	5.5±0.2	6.2±0.3	5.8±0.2	7.9±0.1	7.3±0.2	4.5±0.2
Total Cu[b] (mg/kg)	9.0±0.5	10.6±0.44	26.2±1.7	25.7±1.1	29.1±0.5	8.2±0.3
Texture[c]	loamy sand	sandy loam	silt loam	clay loam	loam	sandy
Clay (%)	5.9	7.0	16.5	33.8	27.1	0.8
Silt (%)	8.9	22.5	53.2	46.1	40.9	2.9
Sand (%)	85.2	70.5	30.3	20.1	32.0	96.3
OM[d] (%)	6.4±0.9	5.7±0.2	21.7±1.3	10.2±0.6	12.3±0.7	2.0±0.4
CEC[e] (cmol/kg)	8.3±0.4	10.1±0.3	38.8±2.2	16.5±1.2	30.9±2.2	1.7±0.3
WHC[f] (%)	36.5±3.3	53.5±4.6	69.3±5.7	47.7±2.9	52.1±4.6	25.1±3.2

[a]pH in 0.01M $CaCl_2$ extract. [b]*aqua regia* digestion. [c]Determined by the hydrometer method (Pansu and Gautheyrou, 2006). [d]Organic matter content determined by the ignition method (Pansu and Gautheyrou, 2006). [e]Cation exchange capacity determined by ammonium acetate method (Pansu and Gautheyrou, 2006). [f]Maximum water-holding capacity determined by the saturation and gravity drainage method (Pansu and Gautheyrou, 2006).

Entire dose−response relationships

The relationships between survival rate of earthworms and three different expressions of exposure (total Cu concentration, $\{Cu^{2+}\}$, andγ_{EFFECT}) are shown in Figure 2.1. Earthworm survival rate decreased with increasing total Cu concentrations (Figure 2.1, first column). Copper toxicity to each earthworm species varied widely in the different soils. When expressed as total concentration, LC50 of Cu ranged from 32.4 to 284 mg/kg for *L. rubellus*, from 39.5 to 1942 mg/kg for *A. longa*, and from 82.8 to 3717 mg/kg for *E. fetida* (Table 2.2). Apart from calculating LC50s in six individual soils, an overall LC50 for each species was also obtained by fitting the toxicity data in all soils together with equation 2-5 (Table 2.2). In case of using the total Cu concentration as the expression for Cu toxicity, poor fits were observed with R^2 of 0.35 and RMSE of 33.9 for *L. rubellus*, R^2 of 0.32 and RMSE of 36.9 for *A. longa*, and R^2 of 0.27 and RMSE of 33.0 for *E. fetida* (Figure 2.1, first column). The large differences between the individual LC50 values and the overall LC50 values showed that total soil concentration failed to explain the variation in Cu toxicity among soils.

When using the computed $\{Cu^{2+}\}$ as dose, the goodness of fit was considerably improved (Figure 2.1, second column). The R^2 and RMSE values were, respectively, 0.79 and 18.7 for *L. rubellus*, 0.89 and 14.1 for *A. longa*, and 0.57 and 25.9 for *E. fetida* (Figure 2.1). LC50$\{Cu^{2+}\}$ for different earthworms in each soil are shown in Table 2.2. There are extensive evidence showing that total concentrations of metals in soils are poor indicators for toxicity and may result in either underestimation or overestimation of the actual risks (Mertens et al., 2007; Peijnenburg et al., 2007; Thakali et al., 2006b; Van Gestel and Koolhaas, 2004), while free ion activities are the immediately available metal fractions and can represent the bioavailability much better (Hobbelen et al., 2006). The results of the

present study confirmed what could be expected: free Cu^{2+} in soil porewater, rather than total Cu in soil, is the dominant toxic species for earthworms.

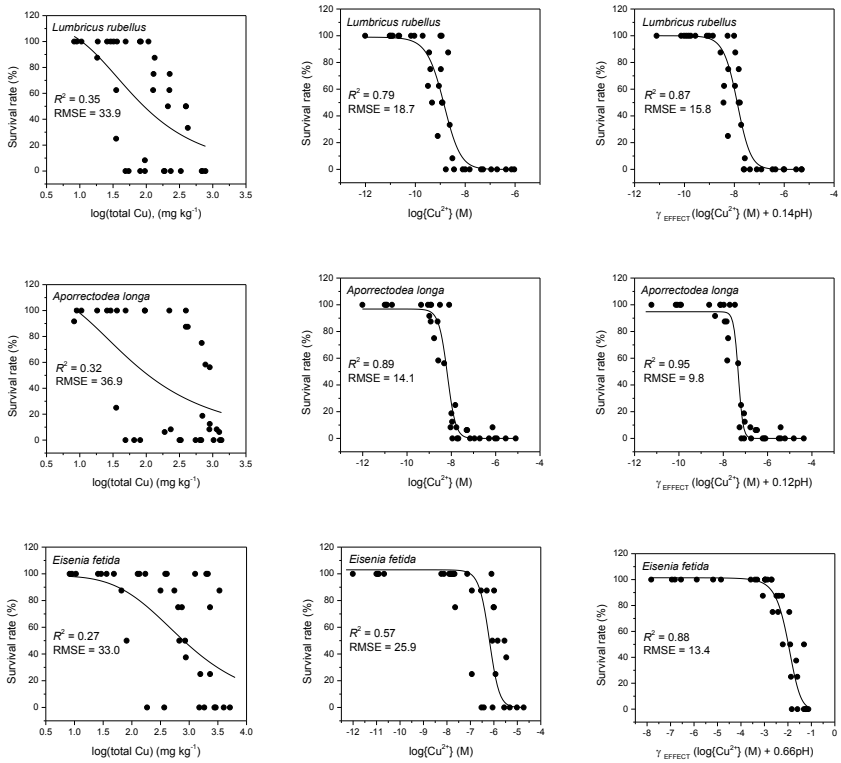

Figure 2.1 Dose−response relationships between survival rate and total Cu concentration in soil (first column), free Cu^{2+} activity in soil porewater (second column), effect dose γ_{EFFECT} (third column) for the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* in all six tested soils after 28 days of exposure. The solid lines represent the log-logistic model fits (equation 2-5). R^2 indicates the coefficient of determination of the linear regression between the predicted and observed survival rate. RMSE indicates root mean square error of the predicted survival rate.

Table 2.2 Median lethal concentrations of Cu expressed as total Cu concentrations (LC50[Cu], (mg/kg)) and free Cu^{2+} activities (LC50{Cu^{2+}}, (M)) for the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* exposed in different soils for 28 days (n = 3).

Soils	LC50[Cu][a] for *L. rubellus*	logLC50{Cu^{2+}} for *L. rubellus*	LC50[Cu] for *A. longa*	logLC50{Cu^{2+}} for *A. longa*	LC50[Cu] for *E. fetida*	logLC50{Cu^{2+}} for *E. fetida*
Boxtel	72.2	-8.65	111.1	-7.89	1322	-6.14
	(64.7~80.4)[b]	(-8.52~-8.80)	(80.2~153.9)	(-7.42~-8.17)	(1033~1692)	(-5.89~-6.31)
Drimmelen	279.2	-8.94	753.2	-8.37	2486	-7.41
	(193.8~402.2)	(-8.82~9.22)	(559.2~1014)	(-8.18~-8.52)	(2390~2586)	(-7.18~-7.67)
Mook	32.4	-7.69	39.5	-6.94	82.8	-5.46
	(28.1~37.3)	(-7.50~-7.83)	(34.9~44.7)	(-6.71~-7.16)	(68.5~100.1)	(-5.24~-5.59)
Valkenswaard	36.1	-8.09	156.8	-7.12	667.0	-5.56
	(30.4~42.9)	(-7.91~-8.23)	(142.5~172.5)	(-6.84~-7.43)	(574.5~774.4)	(-5.33~-5.72)
Vlaardingen	238.3	-9.30	618.0	-8.44	2233	-7.13
	(162.9~348.6)	(-9.11~-9.42)	(548.9~695.7)	(-8.12~-8.70)	(2144~2326)	(-7.01~-7.38)
Woerden	283.6	-8.36	1942	-7.68	3717	-6.05
	(208.4~385.8)	(-8.25~-8.62)	(1779~2119)	(-7.34~-7.81)	(3678~3756)	(-5.85~-6.26)
All Soils	139.1 (NA)[c]	-8.86 (NA)	175.9 (NA)	-7.32 (NA)	1001 (NA)	-6.17 (NA)

[a]Individual LC50 for each species in each soil was calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). The overall LC50 for each species was obtained by fitting the toxicity data in all tested soils together with a logistic dose-response curve (equation 2-5).
[b]95% confidence intervals. [c]Not applicable

When applying the logistic dose−response model to fit all data with the effect dose ($\gamma_{EFFECT} = \log\{Cu^{2+}\}_{EFFECT} - \alpha$ pH), the model provided superior fits in comparison to the fits obtained with other models for *L. rubellus* ($R^2 = 0.87$, RMSE = 15.8), for *A. longa* ($R^2 = 0.95$, RMSE = 9.8), and especially for *E. fetida* ($R^2 = 0.88$, RMSE = 13.4) (Figure 2.1, third column). The effect dose term was then extended to consider an extra cation (Na^+, Ca^{2+}, Mg^{2+}, and K^+) as well as H^+, the corresponding R^2 values for *L. rubellus* were 0.87, 0.85, 0.87, and 0.86, respectively, for *A. longa* were 0.96, 0.97, 0.95, and 0.93, respectively, and for *E. fetida* were 0.89, 0.86, 0.90, and 0.87, respectively. This indicated that the predictive capacity of the model was not enhanced by accounting for the effect of other cations. According to the BLM theory, it was postulated that metal toxicity is dependent on the free ion activity in soil porewater and that cations competition can modify metal toxicity (De Schamphelaere and Janssen, 2002; Thakali et al., 2006a). By accounting for the bioavailability-modifying factor pH only, the application of the effect dose γ_{EFFECT} provided a feasible approach for modelling the entire dose−response curves of Cu toxicity to earthworms.

Species sensitivities

Table 2.2 provides more information on species variation. The species variation was observed in terms of sensitivity (LC50), decreasing in the order: *L. rubellus* > *A. longa* > *E. fetida*. Spurgeon et al. (2000) found that earthworms *L. rubellus* and *A. caliginosa* were more sensitive to Zn than *L. terrestris* and *E. fetida*. Langdon et al. (2005) reported that the sensitivity of three earthworm species to Pb followed the decreasing order: *L. rubellus* > *A. caliginosa* > *E. fetida*. Species-specific differences in ecological strategies and physiological characteristics (detoxification and elimination strategies) might account for the differences in earthworm sensitivity (Morgan et al., 2002; Nahmani et al., 2007). The epigeic *E. fetida* feeds almost entirely on the soil surface on organic matter (cow manure in the present study), whereas endogeic *A. caliginosa* and anecic *A. longa* would be more exposed to metals as they live and feed in the soil (Langdon et al., 2005). In addition, the activity of calcium glands in earthworms may partially account for the differences in sensitivity as calcium involves in the sequestration and elimination of many metals. It was found that the more tolerant species *E. fetida* has more active calcium secretion glands than the other sensitive species (Piearce, 1972; Spurgeon et al., 2000).

Cu toxicity to earthworm was soil and species specific. It is worth to note that although soil factors influenced to different extents the LC50s of Cu for different earthworm species, the relative sensitivity of these species did not change in different soils, that is, all earthworm species tested, irrespective of their ecological characteristics and ecotypes, gave the same ranking in sensitivity to Cu. These findings provided the possibility of extrapolating the results for one earthworm species to other earthworm species, which is significant for further developing a multispecies toxicity model for the purpose of environmental risk assessment.

Prediction of LC50{Cu^{2+}}

Stepwise multiple-linear-regression analysis was used to generate equations to predict the LC50{Cu^{2+}} in relation to soil porewater properties. The soil porewater properties at LC50s for each soil were interpolated from the measured values (Table S2.3−S2.5 in the SI). For all three earthworm species (*L. rubellus*, *A. longa, and E. fetida*), only pH was identified

as the explanatory variable of Cu toxicity and incorporated into the multicomponent Freundlich model (equation 2-2). Other cations were the excluded variables as they did not improve the model fit significantly (when the Freundlich-type model comprising pH and one of the variables Na^+, Ca^{2+}, Mg^{2+}, and K^+, the corresponding R^2 values for *L. rubellus* were 0.80, 0.83, 0.79, and 0.85, respectively, for *A. longa* were 0.92, 0.93, 0.93, and 0.92, respectively, and for *E. fetida* were 0.95, 0.98, 0.98, and 0.98, respectively). The estimated parameters for the final model are given in Table 2.3. In all cases, model parameter α for pH was negative, indicating increased toxicity with increasing pH (i.e., decreasing H^+), which is in agreement with the competitive concept of the BLM. Model parameter α revealed that an increase in pH with one unit results in a 2.6-fold, 4.6-fold and 6.0-fold decrease of LC50{Cu^{2+}} for *L. rubellus*, *A. longa*, and *E. fetida*, respectively. The model obtained explained, respectively, 84%, 94%, and 96% of the variance in logLC50{Cu^{2+}} for *L. rubellus*, *A. longa*, and *E. fetida*.

Table 2.3 Parameter estimates for the multicomponent Freundlich Model (equation 2-2) using the data of the present study on Cu toxicity to earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* in all six soils tested and the literature data on Cu toxicity to other soil invertebrates, plants and microbial processes in a range of European soils. The parameters were estimated by multiple-linear-regression.

	α	γ	R^2_{adj} [h]
L. rubellus	−0.42 (0.09)[g]	−5.84 (0.61)	0.84
A. longa	−0.66 (0.07)	−3.27 (0.47)	0.94
E. fetida	−0.78 (0.07)	−1.24 (0.46)	0.96
ECP[a]	−0.93 (0.09)	−0.94 (0.42)	0.94
FJP[b]	−0.56 (0.08)	−2.35 (0.40)	0.86
HRE[c]	−0.82 (0.07)	−2.46 (0.38)	0.93
LSY[d]	−0.87 (0.09)	−1.99 (0.47)	0.91
GIR[e]	−1.27 (0.24)	1.52 (0.91)	0.79
PNR[f]	−0.69 (0.12)	−2.15 (0.74)	0.74

[a]ECP is *Eisenia fetida* cocoon production (Criel et al., 2008; Thakali et al., 2006b). [b]FJP is *Folsomia candida* juvenile production (Criel et al., 2008; Thakali et al., 2006b). [c]HRE is *Hordeum vulgare* root elongation (Rooney et al., 2006; Thakali et al., 2006a), [d]LSY is *Lycopersicon esculentum* shoot yield (Rooney et al., 2006; Thakali et al., 2006b). [e]GIR is glucose induced respiration (Oorts et al., 2006; Thakali et al., 2006b). [f]PNR is potential nitrification rate (Oorts et al., 2006; Thakali et al., 2006b). [g]Standard errors are indicated in brackets. [h]R^2_{adj} indicates the coefficients of determination adjusted for the degrees of freedom for observed versus predicted values.

The accuracy of model predictions is shown in Figure 2.2A. In general, the model gave good predictions for Cu toxicity to all different species of earthworms tested. Predicted values were in good agreement with the observed values, with an error of less than a factor of 2, indicating that the multicomponent Freundlich can be used to predict Cu toxicity to earthworms irrespective of their ecotypes in a range of soils of varying properties.

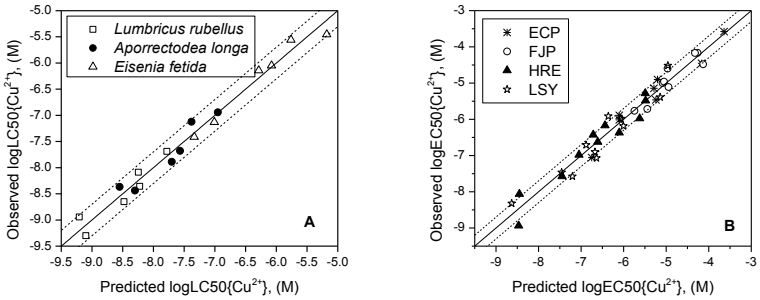

Figure 2.2 Relationships between the observed and predicted logL(E)C50{Cu^{2+}} for the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* in all six tested soils after 28 days of exposure (A), and for ECP, FJP, HRE, and LSY in a range of European soils (B). ECP, FJP, HRE, and LSY are *Eisenia fetida* cocoon production (Criel et al., 2008; Thakali et al., 2006b), *Folsomia candida* juvenile production (Criel et al., 2008; Thakali et al., 2006b), *Hordeum vulgare* root elongation (Rooney et al., 2006; Thakali et al., 2006a), and *Lycopersicon esculentum* shoot yield (Rooney et al., 2006; Thakali et al., 2006b), respectively. The predictions were based on the multicomponent Freundlich model (equation 2-2) using the parameters given in Table 2.3. The solid line represents the 1:1 line, and the dashed lines represent a factor of 2 differences between the observed and predicted values.

To evaluate if the existing BLMs for one earthworm species can be extrapolated toward other earthworm species, the first step is to qualitatively validate if the toxicity-modifying effects (protective cations) are similar for different earthworm species. For *A. caliginosa*, significant competition of H^+ and Na^+ with Cu^{2+} has been observed with mortality as endpoint in a solution-sand system (Steenbergen et al., 2005). For *E. fetida*, only the protective effect of H^+ (not Ca^{2+} and Mg^{2+}) on Cu toxicity was found with cocoon production as endpoint in soil (Thakali et al., 2006b). In the present study, the same Cu toxicity-modifying factor (pH) was identified for *L. rubellus*, *A. longa*, and *E. fetida*, which was consistent with the findings of Thakali et al. (2006b). The extrapolation of the results from solution to soil is difficult as the exposure is different and soil processes are far more complex than the simple solution. A structural underestimation of toxicity was found when directly applying the model developed from solution-sand system to soils (Koster et al., 2006). The multicomponent Freundlich model in which the competitive effects of pH were included, well predicted logLC50{Cu^{2+}} for all the test species. With regard to the physical meaning of the model parameters, the exponent n_{Cu} (equation 2-1) reflects the sensitivity of species to Cu. It could probably be used as an indicator of toxic strength of metals when different metals are assessed. The exponents n_H and n_{Ci} (equation 2-1) describe to what extent Cu interactions with the earthworms is dependent on pH and on other cations. These parameters are soil and organism specific as the amount and the type of binding sites will vary (Plette et al., 1999). The deduced coefficients α (= n_H/n_{Cu}) and β_i (= n_{Ci}/n_{Cu}) (equation 2-2) quantify the competition of H^+ and other cations with Cu^{2+} at the site of toxic action. Thus, the α values differed for different species, suggesting that the effects of pH on Cu toxicity

differed per species. In our study, the obtained α values did reflect the actual sensitivity of earthworms to Cu by taking the toxicity-modifying factors into account and the γ values were constants at the given effect level.

In the literature, no consistent effects of cations (K^+, Na^+, Ca^{2+}, and Mg^{2+}) on Cu toxicity are found. Protective effects of H^+, Ca^{2+}, and Mg^{2+} against Cu toxicity to bioluminescence of *Nitrosomonas europaea* were observed in a study by Ore et al.(2010). Le et al. (2012) developed a BLM to estimate Cu toxicity to lettuce (*Lactuca sativa*) in terms of root elongation. They found that only H^+ can be integrated into the BLM, and competitive effects of the cations (K^+, Na^+, Ca^{2+}, and Mg^{2+}) were insignificant. A protective effect of Ca^{2+} and Mg^{2+} and no effect of H^+, Na^+ and K^+ were found for wheat root (Luo et al., 2008). The appearance of the inconsistencies might be explained from the following aspects. First, it is most likely that binding constants for different cations differ across species. Second, the covariance between H^+ and other cations may mask the effects of cations competition in soil solution. Such covariance was observed in the study of Thakali et al. (2006a; 2006b). They showed that major cations (such as Ca^{2+} and Mg^{2+}) tend to increase with increasing Cu concentrations and decreasing pH. Third, the difference in the endpoints used is a possible explanation. For example, an obvious effect on reproduction does not mean a visible effect on the mortality (Santorufo et al., 2012). Even ranking of species sensitivity can change with the endpoints selected. Last, the narrow range of dissolved cations concentrations in the porewater of the soils tested may also limit the assessment of the effects of cations on Cu toxicity.

Extrapolation of study results to other studies

An important question in generalizing the toxicity model within a regulatory framework is whether or not the model developed for one species can be extrapolated to other species with different endpoints. In case of Cu, there are existing data sets (Criel et al., 2008; Oorts et al., 2006; Rooney et al., 2006; Thakali et al., 2006a; 2006b) that are suitable for external validation of the applicability of the multicomponent Freundlich model. These data sets consist of six toxicity tests, covering soil invertebrates, plants and microbial processes, in a range of European soils. The levels of K^+, Na^+, Ca^{2+}, and Mg^{2+} in soil porewater (Criel et al., 2008; Oorts et al., 2006; Rooney et al., 2006; Thakali et al., 2006a; 2006b) were comparable to that of the present study. It should be realized that when other studies and other species were used, they may be exposed to different soils with different properties. Previously, these toxicity data were used for developing Cu-BLMs (Thakali et al., 2006a; 2006b) and a pH dependence of Cu^{2+} toxicity was found for all species. In the present study, equation 2-2 with only pH of the soil porewater as the explanatory variable was applied to the underlying data from the literature. Table 2.3 shows the estimated model parameters for *E. fetida* cocoon production (ECP), *Folsomia candida* juvenile production (FJP), *Hordeum vulgare* root elongation (HRE), and *Lycopersicon esculentum* shoot yield (LSY). The pH exerted similar effects on Cu toxicity for ECP, HRE and LSY as reflected by the parameter α. More than 86% of the variation in toxicity was explained by the models for all these species. Nearly all the predicted values of $\log EC50\{Cu^{2+}\}$ were within a factor of 2 of the observed values (Figure 2.2B). This level of precision in prediction of EC50 is similar to that of the BLM developed by Thakali et al. (2006a; 2006b). For the microbial processes glucose induced

respiration (GIR) and potential nitrification rate (PNR), the toxicity variances explained by the model were 79% and 74%, respectively (Table 2.3). The model performed better in predicting Cu toxicity to soil invertebrates and plants than to soil microorganisms. This finding could be explained by the varying microbial communities among soils and the fact that they are less mobile as soil microorganisms are usually attached to the soil particles, suggesting that the microbe bathing solution may differ from the porewater that can often be used to assess metal bioavailability for soil invertebrates and plants (Mertens et al., 2007; Ore et al., 2010). Therefore, care should be taken when extrapolating the results to microbial processes. The generalized model ($\log L(E)C50\{Cu^{2+}\} = \alpha\ pH + \gamma$) accurately predicted toxicity for soil invertebrates and plants across different soils, suggesting that the same processes of the soil (i.e., the impact of pH) played a role in ranking Cu toxicity for these soil organisms and that the model can be extrapolated to other soil organisms except for microorganisms.

2.4 Implications

In the context of implementing the use of bioavailability models into environmental risk assessment of Cu for soil organisms, it is important to assess the effects of the variability of porewater chemistry parameters on Cu toxicity across different species. To produce a theoretical function for soil Cu toxicity, a terrestrial BLM is an option. However, unlike the case of a hydroponic system, it is difficult to provide direct evidence for the protective effects of cations in soils. The prerequisite of the BLM in requiring linear relations between the protective effects of the cations and metal toxicity was not met by the results of the present study. The multicomponent Freundlich model, requiring fewer parameters than the BLM, proved to be a feasible framework for directly linking the porewater chemistry to Cu toxicity in soils for various soil dwelling organisms and plants with different endpoints. Based on the results of our study, the developed models can be used to evaluate environmental risks associated with the specific Cu-contaminated soil for the corresponding species as long as the Cu^{2+} activity and pH in the soil porewater are known. In practice, soil solutions tend to exhibit negative covariance of pH and other cations since pH is controlled by the concentrations of the other ions (Lofts et al., 2004; Thakali et al., 2006b; Wang et al., 2011a). Although Na^+, Ca^{2+} and Mg^{2+} may be important factors in modifying metal toxicity by means of competition, the equation ($\log LC50\{Cu^{2+}\} = \alpha\ pH + \gamma$) does not imply that the H^+ alone exerts a protective effect but rather that the overall protective effect of all free cations may be expressed as a function of pH alone (Lofts et al., 2004). It is thus possible that the need to consider the effects of other cations implicitly rather than explicitly is not a source of significant uncertainty in the model derived. Even so, for future users of the model, it is suggested that where free cation activities (especially Ca^{2+} and Mg^{2+}) are controlled by solid phases, an explicit consideration of these cations (whether or not to be included in the extended model) may be appropriate. In those cases, the Freundlich-type model can be easily extended to incorporate the effect of those cations ($\log LC50\{Cu^{2+}\} = \alpha\ pH - \sum \beta_i \log\{C_i^{z+}\} + \gamma$). Thus, the extrapolation is possible in both cases.

Supporting information

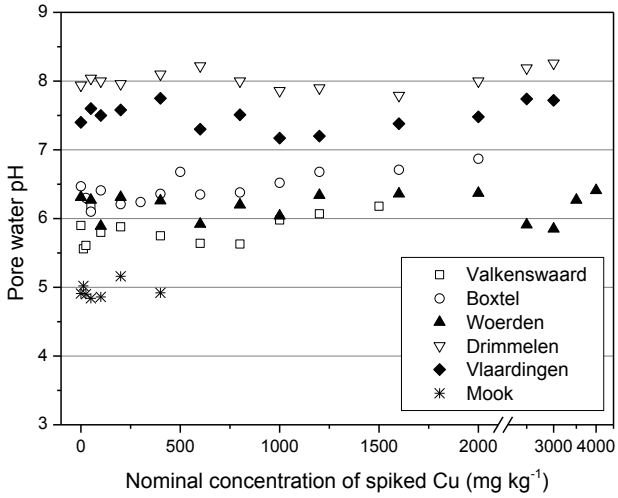

Figure S2.1 Relationships between soil porewater pH and nominal concentrations of Cu spiked into the soils tested. For soil Valkenswaard, 12 levels of Cu concentrations (0-1500 mg/kg) were spiked; for soil Boxtel, 14 levels of Cu concentrations (0-2000 mg/kg) were spiked; for soil Woerden, 15 levels of Cu concentrations (0-4000 mg/kg) were spiked; for soil Drimmelen and soil Vlaardingen, 13 levels of Cu concentrations (0-3000 mg/kg) were spiked; for soil Mook, 8 levels of Cu concentrations (0-400 mg/kg) were spiked.

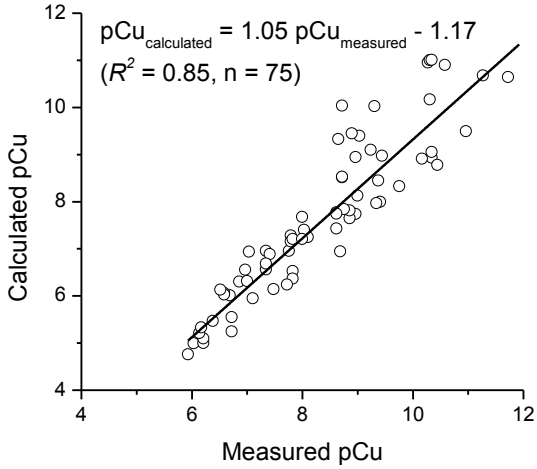

Figure S2.2 Relationship between the calculated free Cu^{2+} activity (expressed as pCu) by the Windermere Humic-Aqueous Model and the measured pCu by copper ion-selective electrode in the porewater of all six tested soils.

Table S2.1 Selected properties of the porewater of unspiked soils (Valkenswaard, Boxtel, Woerden, Drimmelen, Vlaardingen, and Mook). All values are given as means of three replicates.

	Valkenswaard	Boxtel	Woerden	Drimmelen	Vlaardingen	Mook
pH	5.8	6.4	6.2	8.0	7.4	5.0
Dissovled Cu (mg/L)	0.07	0.03	0.05	0.04	0.03	0.04
DOC[a] (mg/L)	149.4	181.7	502.5	192.3	342.4	188.6
Dissovled Na (mg/L)	41.3	66.1	119.1	97.4	45.0	43.8
Dissovled K (mg/L)	190.9	68.8	39.0	15.3	31.7	96.8
Dissovled Ca (mg/L)	120.3	198.7	509.2	369.7	336.7	101.0
Dissovled Mg (mg/L)	92.6	71.5	141.0	56.3	51.5	79.4

[a]Dissolved organic carbon determined with TOC analyzer (TOC-VCSH, Shimadzu)

Table S2.2 Linear regression relationships between dissolved Cu concentrations in soil porewater (Cu_{pw}, mg/L), total soil Cu concentrations (Cu_{total}, mg/kg), pH of the porewater, and dissolved organic carbon (DOC, mg/L) in the six tested soils

Soils	Equations	R^2_{adj} [a]	n	F	p
Valkenswaard	$\log Cu_{pw} = 0.84\ (\pm0.04) \log Cu_{total} - 1.88\ (\pm0.09)$	0.978	12	507.7	<0.0001
	$\log Cu_{pw} = 0.84\ (\pm0.04) \log Cu_{total} - 0.04\ (\pm0.05)\ pH - 1.63\ (\pm0.29)$	0.978	12	251.1	<0.0001
	$\log Cu_{pw} = 0.91\ (\pm0.04) \log Cu_{total} - 0.08\ (\pm0.04)\ pH + 0.002\ (\pm0.0006)\ DOC - 1.82\ (\pm0.24)$	0.986	12	272.9	<0.0001
Boxtel	$\log Cu_{pw} = 1.01\ (\pm0.04) \log Cu_{total} - 2.49\ (\pm0.10)$	0.979	14	630.4	<0.0001
	$\log Cu_{pw} = 1.04\ (\pm0.04) \log Cu_{total} - 0.16\ (\pm0.06)\ pH - 1.52\ (\pm0.41)$	0.985	14	444.6	<0.0001
	$\log Cu_{pw} = 0.99\ (\pm0.05) \log Cu_{total} - 0.20\ (\pm0.07)\ pH + 0.001\ (\pm0.0008)\ DOC - 1.32\ (\pm0.41)$	0.986	14	325.9	<0.0001
Woerden	$\log Cu_{pw} = 1.09\ (\pm0.06) \log Cu_{total} - 3.16\ (\pm0.22)$	0.941	15	222.7	<0.0001
	$\log Cu_{pw} = 1.04\ (\pm0.07) \log Cu_{total} - 0.12\ (\pm0.07)\ pH - 2.25\ (\pm0.57)$	0.948	15	128.7	<0.0001
	$\log Cu_{pw} = 0.92\ (\pm0.05) \log Cu_{total} - 0.06\ (\pm0.05)\ pH + 0.0004\ (\pm0.0001)\ DOC - 2.53\ (\pm0.36)$	0.979	15	229.0	<0.0001
Drimmelen	$\log Cu_{pw} = 0.84\ (\pm0.07) \log Cu_{total} - 2.45\ (\pm0.19)$	0.926	13	153.2	<0.0001
	$\log Cu_{pw} = 0.83\ (\pm0.07) \log Cu_{total} - 0.34\ (\pm0.31)\ pH - 5.12\ (\pm2.44)$	0.928	13	78.7	<0.0001
	$\log Cu_{pw} = 0.75\ (\pm0.03) \log Cu_{total} - 0.15\ (\pm0.13)\ pH + 0.002\ (\pm0.0002)\ DOC - 1.33\ (\pm1.02)$	0.990	13	402.7	<0.0001
Vlaardingen	$\log Cu_{pw} = 1.04\ (\pm0.07) \log Cu_{total} - 2.82\ (\pm0.20)$	0.948	13	220.3	<0.0001
	$\log Cu_{pw} = 1.04\ (\pm0.07) \log Cu_{total} - 0.10\ (\pm0.14)\ pH - 2.09\ (\pm1.07)$	0.946	13	105.2	<0.0001
	$\log Cu_{pw} = 0.91\ (\pm0.03) \log Cu_{total} - 0.14\ (\pm0.04)\ pH + 0.0009\ (\pm0.0001)\ DOC - 1.74\ (\pm0.33)$	0.994	13	760.1	<0.0001
Mook	$\log Cu_{pw} = 0.92\ (\pm0.09) \log Cu_{total} - 1.40\ (\pm0.16)$	0.936	8	104.0	<0.0001
	$\log Cu_{pw} = 0.99\ (\pm0.08) \log Cu_{total} - 0.19\ (\pm0.10)\ pH - 0.56\ (\pm0.45)$	0.956	8	77.7	<0.0001
	$\log Cu_{pw} = 1.08\ (\pm0.06) \log Cu_{total} - 0.24\ (\pm0.07)\ pH + 0.001\ (\pm0.0004)\ DOC - 0.70\ (\pm0.29)$	0.982	8	129.9	<0.0001

[a] R^2_{adj} is the coefficient of determination adjusted for the degrees of freedom; n indicates the number of data points; F is the value of F test; p indicates the statistical significance level; Standard errors are indicated in brackets.

Table S2.3 The soil porewater properties (pH, dissolved organic carbon (DOC), and dissolved cations concentrations) interpolated at LC50 values of Cu (expressed as total soil concentration) for *Lumbricus rubellus* in the six soils tested. The function TREND in Microsoft Excel 2010 was used for interpolation based on the measured values.

Soil	LC50 (mg/kg)	Porewater pH	DOC (mg/L)	Dissolved cations in soil porewater (mg/L)				
				Ca	Mg	Na	K	Cu
Boxtel	72.2	6.39	161.4	252.5	74.5	73.4	66.3	0.29
Drimmelen	279.2	8.00	168.9	419.2	69.2	103.4	15.6	0.50
Mook	32.4	4.95	220.8	138.2	92.8	53.5	137.8	0.94
Valkenswaard	36.1	5.98	186.3	105.9	69.5	46.4	171.7	0.29
Vlaardingen	238.3	7.54	260.0	332.5	65.9	41.1	33.0	0.63
Woerden	283.6	6.21	323.4	526.0	161.0	118.3	43.1	0.28

Table S2.4 The soil porewater properties (pH, dissolved organic carbon (DOC), and dissolved cations concentrations) interpolated at LC50 values of Cu (expressed as total soil concentration) for *Aporrectodea longa* in the six soils tested. The function TREND in Microsoft Excel 2010 was used for interpolation based on the measured values.

Soil	LC50 (mg/kg)	Porewater pH	DOC (mg/L)	Dissolved cations in soil porewater(mg/L)				
				Ca	Mg	Na	K	Cu
Boxtel	111.1	6.43	176.9	267.9	82.4	77.5	71.2	0.50
Drimmelen	753.2	8.03	263.4	438.3	76.8	111.0	17.7	1.22
Mook	39.5	4.95	226.9	147.6	102.8	57.8	141.9	1.23
Valkenswaard	156.8	5.66	171.5	182.2	125.0	66.1	234.1	0.92
Vlaardingen	618.0	7.44	423.2	437.6	92.8	56.1	37.7	1.77
Woerden	1942	6.19	533.1	496.3	152.4	119.4	39.0	2.90

Table S2.5 The soil porewater properties (pH, dissolved organic carbon (DOC), and dissolved cations concentrations) interpolated at LC50 values of Cu (expressed as total soil concentration) for *Eisenia fetida* in the six soils tested. The function TREND in Microsoft Excel 2010 was used for interpolation based on the measured values.

Soil	LC50 (mg/kg)	Porewater pH	DOC (mg/L)	Dissolved cations in soil porewater (mg/L)				
				Ca	Mg	Na	K	Cu
Boxtel	1322	6.64	217.6	198.7	57.8	69.8	63.7	3.87
Drimmelen	2486	7.94	196.0	341.2	36.2	86.8	13.2	2.10
Mook	82.8	5.05	218.4	100.9	143.9	54.4	140.4	2.31
Valkenswaard	667.0	5.72	130.8	120.3	112.1	62.3	147.5	3.07
Vlaardingen	2233	7.49	369.0	336.6	45.7	39.3	29.5	3.56
Woerden	3717	6.17	559.0	420.9	136.5	122.8	38.6	6.14

Chapter III

Modelling Cadmium And Nickel Toxicity To Earthworms With The Free Ion Approach

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Abstract

The use of the Free Ion Approach to quantify the toxic effects of Cd and Ni to the earthworms *Lumbricus rubellus* and *Aporrectodea longa* exposed in soils of different types was explored. Median lethal concentration (LC50) of Cd (expressed as the total concentration in soil) varied by approximately 11 and 28 fold for *L. rubellus* and *A. longa*, respectively. For Ni, these values were 50 and 38, respectively. For the two earthworm species, no significant influence of cations (H^+, Ca^{2+}, Mg^{2+}, K^+, and Na^+) on Cd^{2+} toxicity was observed, while Mg^{2+} was found to significantly alleviate Ni^{2+} toxicity. The Free Ion Activity Model, which is a special case of the Free Ion Approach with no impact of cations, sufficiently described the variability in Cd^{2+} toxicity across soils but failed in predicting Ni^{2+} toxicity. The Free Ion Approach, in which the protective effects of Mg^{2+} were included, explained 89% and 84% of the variations in LC50{Ni^{2+}} (expressed as free ion activity) for *L. rubellus* (logLC50{Ni^{2+}} = 1.18log{Mg^{2+}} − 0.52) and *A. longa* (logLC50{Ni^{2+}} = 0.51log{Mg^{2+}} − 2.16), respectively. Prediction error was within a factor of 2 for both Cd^{2+} and Ni^{2+} toxicity, indicating the applicability of the Free Ion Approach for predicting toxicity of these two metals. Although extrapolation of the Free Ion Approach across metals still needs more research efforts, this approach, as an alternative for the biotic ligand model, provides a feasible framework for site-specific risk assessment.

3.1 Introduction

Toxicity of metals to soil organisms frequently deviates across different soil types. For example, up to a factor of 11 and 50 differences in median effect concentrations (EC50) of Cu were found for *Eisenia fetida* cocoon production and *Folsomia candida* juvenile production, respectively, in 19 European soils (Criel et al., 2008). The EC50 of Ni for barley root elongation reached a maximum variation of 37-fold in 16 soils (Rooney et al., 2007). Data interpretation is complicated as the effects are metal- or species-specific. Furthermore, many soil properties are more or less correlated with each other (Wang et al., 2011a), making it difficult to distinguish the real source of an effect from the interdependent factors. Many empirical models have been developed to relate metal toxicity to typical soil properties, such as pH, organic matter content, and cation exchange capacity (CEC) (Criel et al., 2008; Oorts et al., 2006; Rooney et al., 2007). The predictive ability of these models seems to be reasonably well. However, the mechanisms underlying such empirical relationships are not fully revealed.

In the past years, many studies have been performed attempting to understand the exact mechanisms that induce differences in bioavailability and toxicity. An important development in this respect is the finding that the free metal ion is the dominant species that can be taken up by the organisms and subsequently induces toxicity. This triggered the development of the free ion activity model (FIAM) (Campbell, 1995; Morel, 1983). After it was shown that uptake and effect of a metal are highly influenced by other coexisting cations (e.g., H^+, Ca^{2+}, and Mg^{2+}), the FIAM was extended into the biotic ligand model (BLM) in order to incorporate both metal speciation and the effects of competition with other cations (De Schamphelaere and Janssen, 2002; Plette et al., 1999). The BLM concept is based on the mechanistic assumption that metal toxicity depends on the amount of metal ion bound to active sites within the organism (i.e., biotic ligand, BL), while protons and other cations can alleviate metal toxicity by competitive binding to the BL (De Schamphelaere and Janssen, 2002).

In the aquatic environment, the BLM has shown the advantage of being generally valid and applicable, as it relies on fundamental thermo-dynamical rather than fitting parameters (Di Toro et al., 2001). However, the applicability of BLM theory to soils has rarely been evaluated. Several BLMs have been developed for soil animals such as a Cu-BLM for the earthworm *Aporrectodea caliginosa* (Steenbergen et al., 2005), a Cd-BLM for the earthworm *Eisenia fetida* (Li et al., 2008), and a Co-BLM for the enchytraeid *Enchytraeus albidus* (Lock et al., 2006). BLMs were also used to assess the effects of metals on plants (Li et al., 2009a; Lock et al., 2007). All these BLMs were developed using solution (or solution-sand) as exposure medium instead of real soil, as it is difficult to univariately manipulate soil porewater properties and to estimate reliable and representative BLM parameters. Thakali and co-workers (Thakali et al., 2006a; 2006b) reported the development and application of BLMs for predicting Cu and Ni toxicity to invertebrates, plants and microbial processes in soils. They found that it is always necessary to empirically fix one or more parameters of the BLM before fitting the binding constants for each cation. Therefore, the derived stability constants should not be regarded as conventional stability constants but rather as parameters that summarize the processes underlying the observed relationships between metal toxicity and protective cations.

Recently, based on empirical studies and BLM theory, Lofts and co-workers (Lofts et al., 2004; 2013) have proposed an alternative method, the Free Ion Approach to describe the variation in the toxic effects of a metal among soils of varying compositions, with the variation in soil porewater pH and concentrations of other protective cations (e.g., Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}). This approach, requiring fewer parameters than the BLM, has been successfully used to model Cu toxicity to soil invertebrates (including earthworms) and plants (Lofts et al., 2013; Qiu et al., 2013). However, the applicability for other metals is still unknown. In addition, a lack of consistent effects caused by other cations is often found between solution cultures and soil cultures. For example, in hydroponic systems, pH and Na^{+} were reported to alleviate Cu toxicity to *A. caliginosa* (Steenbergen et al., 2005). In a soil matrix, however, only a pH dependence of Cu^{2+} toxicity (no protective effects of other cations) was found for *Lumbricus rubellus*, *Aporrectodea longa* and *E. fetida* (Qiu et al., 2013). More soil toxicity data sets are therefore needed for the purpose of comparison and reliable extrapolation.

The aims of the present study were to investigate the applicability of the Free Ion Approach for predicting Cd and Ni toxicity to *Lumbricus rubellus* and *Aporrectodea longa* in a range of soils of varying compositions, and to examine whether the toxicity-modifying factors are similar for these two earthworm species.

3.2 Materials and methods

Test organisms

The earthworms *Lumbricus rubellus* and *Aporrectodea longa* were used as test organisms. They are natural species and showed to be more sensitive to metals than the artificially cultured *E. fetida* (Qiu et al., 2013; Spurgeon et al., 2000). *L. rubellus* (epigeic) is commonly found throughout Europe and North America. It feeds on surface litter and is found intimately associated with plant roots (Domínguez, 2004). *A. longa* (anecic) is usually present in alkaline soils in open areas such as gardens, grassland and cultivated soils. It draws organic matter from the soil surface into its deep, permanent burrows to feed on (Eisenhauer et al., 2008). Mature *L. rubellus* and *A. longa* were collected from an unexploited grassland in Leiden, The Netherlands. Prior to the toxicity tests the earthworms were cultured in the unspiked soils for at least 1 wk at 15 ± 2 °C to get adapted to the experimental conditions.

Soils and metal spiking

Six different soil samples (coded NL1, NL2, NL3, NL4, NL5, and NL6) were collected from different sites in the Netherlands. The soils were air dried, sieved (< 2mm), and homogenized before use. Several metal concentrations including controls were obtained by spiking the soils with metal acetates (Acros Chemicals; purity > 98%). The spiked Cd concentrations ranged from 25 mg/kg to the maximum of 2500 mg/kg depending on the soils, the spiked Ni concentrations ranged from 50 mg/kg to the maximum of 6600 mg/kg. The spiked soils were left for equilibration for two months before testing, assuring mineralisation of the acetate added (Qiu et al., 2011).

Toxicity tests

The 28-d toxicity tests were performed in a climate chamber at 15 ± 2 °C with a 16h-light: 8h-dark cycle. Adult earthworms with a clearly developed clitellum were used. Earthworms with body weights between 700 and 900 mg for *L. rubellus*, and between 800 and 1000 mg for *A. longa* were selected for testing. Four earthworms were exposed in a plastic jar containing 500 g dry weight of soil. For each treatment, three replicates were used. Soil maximum water-holding capacity was determined by the saturation and gravity drainage method (Pansu and Gautheyrou, 2006). All soils were maintained at 80% maximum water-holding capacity throughout the test by reweighing the test jars periodically and replenishing water loss with deionized water. Earthworms were fed once a week. Approximately 5 g of cow manure was spread on the soil surface of each jar and moistened with 5 mL deionized water. Mortality of the earthworms was scored after 28 d of exposure.

Chemical analysis

Total metal (Cd and Ni) concentrations in soil samples were determined after digestion using a 1:4 v/v mixture of HCl (37% pro analysis, Baker) and HNO_3 (65% pro analysis; Riedel-de-Haen). Soil porewater was collected by suction and subsequent filtration over a 0.45 µm acetate filter of soil samples that were kept at 100% of their maximum water-holding capacity for 1 wk at 15 °C (Qiu et al., 2013). Soil pH (in 0.01M $CaCl_2$ extract) and porewater pH were measured using a pH meter (691, Metrohm AG). Soil properties such as organic matter content (OM), texture, and cation exchange capacity (CEC) were determined following the procedures described by Pansu and Gautheyrou (2006). Dissolved organic carbon (DOC) in soil porewater was measured with a TOC/DOC analyzer (TOC-VCSH, Shimadzu). Concentrations of dissolved metals, Ca, Mg, Na, and K were analysed by flame atomic absorption spectrophotometry (Perkin Elmer AAnalyst 100). The certified reference material ISE 989 (River Clay, Wageningen Evaluating Programs) was used to ensure the accuracy of the analytical procedure for soil digests. Measured metal concentrations were within 15% of the certified reference values.

Data analysis

Metal concentrations inducing 50% mortality (LC50s) of earthworms in each soil were calculated by Probit analysis using SPSS 19.0 (IBM). The soil porewater properties (pH, DOC, and dissolved cations concentrations) corresponding to the total soil metal concentration at the LC50 value (denoted LC50[M]) for each soil were interpolated from the measured values at the tested exposure concentrations. The function TREND in Microsoft Excel 2010 was used for interpolation as generally there were linear relationships between the amount of metal spiked and pH, DOC, and concentrations of dissolved cations in soil porewater. Activities of the free metal ion and of other cations (Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}) in soil porewater were calculated using the speciation model WHAM (VI) (Tipping, 1998). Input parameters included porewater pH, concentrations of dissolved metal and other elements (Ca, Mg, Na, K, Cl^{-}, and SO_4^{2-}), and colloidal fulvic acid (FA). The latter was estimated from the DOC concentration assuming that 65% DOC was active as colloidal FA and 35% DOC was inert for chemical binding (Tipping et al., 2003). In addition, Cl^{-} and SO_4^{2-} in a molar ratio of 6:1 were assumed to maintain electroneutrality (Thakali et al., 2006a; 2006b). Previous studies used the same ratio indicating that the calculated free metal

ion activity was insensitive to the ratio of Cl^- to SO_4^{2-} and that this approximation is unlikely to induce significant uncertainty since the strong acid anions do not enter into significant complexation reactions with Cd and Ni (Qiu et al., 2013; Tipping et al., 2003). Measured values were used for all data analysis unless otherwise indicated.

Free ion approach

The free ion approach is an empirical expression attributing the variation in the toxic effect of a metal, among soils of varying composition, to the variation in soil porewater pH and the variation in concentrations of other solution cations (e.g., Ca^{2+}, Mg^{2+}, K^+, and Na^+):

$$\log\{M^{2+}\}_{LC50} = \alpha\, pH_{pw} + \sum_1^i \beta_i \log\{X_i^{z+}\} + \gamma \quad (3\text{-}1)$$

In equation 3-1, pH_{pw} is the soil porewater pH, $\{X_i^{z+}\}$ (mol/L) is the activity of protective cation in soil porewater, α, β_i, and γ are constants, $\{M^{2+}\}_{LC50}$ (mol/L) is the activity of the free metal ion in soil porewater inducing 50% mortality of organisms. The free ion approach considers the same parameters as would be considered by the BLM. Instead of complex parameter estimation, this can be regarded as a simplification by replacing the BLM with a linear equation that contains only the most sensitive parameters (Qiu et al., 2013; Verschoor et al., 2012). Stepwise multiple linear regression was performed to decided which parameters (H^+, Ca^{2+}, Mg^{2+}, K^+, and Na^+) need to be included into the model. The parameters were included if they passed stepping criteria ($p < 0.05$ for entry, and $p > 0.1$ for removal) and were significant at the probability level $p < 0.001$.

To extend the free ion approach to consider the entire dose-response curves, equation 3-1 can be rearranged and generalized as follows:

$$\gamma_{effect} = \log\{M^{2+}\}_{effect} - \alpha\, pH_{pw} - \sum_1^i \beta_i \log\{X_i^{z+}\} \quad (3\text{-}2)$$

In equation 3-2, γ_{effect} (dimensionless) can be regarded as the effective dose which considers not only the toxicity-driving factor $\{M^{2+}\}$ (mol/L) but also the toxicity-modifying factors $\{X_i^{z+}\}$ (mol/L). γ_{effect} is constant at a given effect level and varies according to the level of effect being described. $\{M^{2+}\}_{effect}$ is the corresponding $\{M^{2+}\}$ at any given effect level.

When the free ion approach was proposed, ion binding was not explicitly considered. To provide the free ion approach with a theoretical basis, a multicomponent Freundlich model was proposed to relate the effective dose term to metal bound to biotic ligand (Qiu et al., 2013):

$$[MBL] = k\, \{M^{2+}\}^{n_M}\, \{H^+\}^{n_H} \prod_1^i \{X_i^{z+}\}^{n_{Xi}} \quad (3\text{-}3)$$

where [MBL] is the amount of metal bound to the BL, k, n_M, n_H, and n_{Xi} are the Freundlich parameters. Rearranging equation 3-3 after log-transformation yields:

$$\log\{M^{2+}\} = \frac{n_H}{n_M} pH + \sum_1^i \frac{n_{Xi}}{n_M} \log\{X_i^{z+}\} + \frac{1}{n_M} \log\left(\frac{[MBL]}{k}\right) \quad (3\text{-}4)$$

If $\frac{n_H}{n_M} = \alpha$, $\frac{n_{Xi}}{n_M} = \beta_i$, and $\frac{1}{n_M} \log\left(\frac{[MBL]}{k}\right) = \gamma_{effect}$ (assuming [MBL] is constant at a given effect level based on BLM theory), equation 3-4 has the same form as equation 3-2. Thus, the effective dose term can be quantitatively linked to metal bound to the biotic ligand of the organism.

A logistic dose-response equation was used to fit the entire toxicity data for each metal:

$$R = \frac{R_0}{1+\left(\frac{X}{X50}\right)^{\beta}} \quad (3\text{-}5)$$

where R is the survival rate, R_o is the control survival rate, β is the slope parameter. When using the free ion approach, $X = \gamma_{effect}$ and X_{50} is the effective dose (dimensionless) causing 50% mortality of the earthworms. The effective dose term can include one or more of the protective cations (H^+, Ca^{2+}, Mg^{2+}, K^+, and Na^+) as well as $\{M^{2+}\}$. The free ion approach is an extension of the free ion activity model by taking the toxicity-modifying factors into account. When no protective effects of cations were found and the γ_{effect} only contained $\{M^{2+}\}$, the free ion approach had the same form as the FIAM. In this case, $X = \{M^{2+}\}$ and X_{50} is the overall $\{M^{2+}\}_{LC50}$. When substituting equation 3-2 into equation 3-5, R was plotted against X to fit the parameters β, X_{50}, and the coefficients for the toxicity-modifying factors. Model parameters were determined by multiple nonlinear regression analysis using SYSTAT 12. Whether the model is significantly improved by the addition of an extra variable is determined by the Akaike information criterion (AIC) (Akaike, 1981). The AIC value deals with the trade-off between the complexity (number of parameters) of the model and the goodness-of-fit of the model. From a statistical viewpoint, the model with the smallest AIC value is preferable.

3.3 Results

Selected properties of soil and porewater

The selected soil and porewater properties of the nonspiked soils are shown in Table 3.1. These soils represented different soil types and varied substantially in pH, OM content, and CEC. Appreciable ranges of DOC and dissolved major cation (Ca, Mg, Na, and K) concentrations in the porewater of the study soils were observed (Table 3.1). The addition of metal salts to the soils resulted in an increase in the Cd or Ni concentration in the soil porewater. The porewater Cd or Ni concentrations correlated linearly with the corresponding total Cd or Ni concentrations in each soil ($p < 0.001$; Table 3.2-3.3). Generally, adding porewater pH as explanatory variable improved R^2 values and the significance of all the regression equations used for predicting metal partitioning between soil solid phase and porewater (Table 3.2-3.3). This is consistent with the metal partitioning being pH-dependent. Significant correlations between a fair amount of parameters in soil porewater were found (Supporting Information, Table S3.1-S3.2). For instance, dissolved Ca and Mg, as well as Ca and Na concentrations were strongly correlated with each other in both Cd-spiked and Ni-spiked soils.

Dose−response relationships for Cd toxicity

Figure 3.1 shows the effect of Cd on the survival of *L. rubellus* and *A. longa*. Control survival of the two species in the nonspiked soils was more than 90% after 28 d of exposure. The earthworm survival rate decreased with increasing total Cd concentration in soil. When expressed as the total soil concentration, the LC50[Cd] ranged between 37 and 401 mg/kg (a variation of 11-fold) for *L. rubellus*, and between 49 and 1409 mg/kg (a variation of 28-fold) for *A. longa* (Table 3.4). *L. rubellus* was more sensitive to Cd than *A. longa* in all tested soils. When applying the total soil Cd concentration to predict Cd toxicity among different soils,

the model fits were poor (R^2 = 0.38 and RMSE = 28.4 for *L. rubellus*; R^2 = 0.24 and RMSE = 28.6 for *A. longa*) (Figure 3.1A and 3.1B). When expressed as $\{Cd^{2+}\}$ in soil porewater, LC50$\{Cd^{2+}\}$ differed by a factor of 2.6 for *L. rubellus* and 2.3 for *A. longa* between the different test soils (Table 3.4). Considerable improvements of the model fits were observed when using $\{Cd^{2+}\}$ as the descriptor for Cd toxicity. The resulting values of R^2 and RMSE were, respectively, 0.94 and 9.5 for *L. rubellus*, and 0.86 and 14.4 for *A. longa* (Figure 3.1C and 3.1D).

Table 3.1 Selected characteristics of the nonspiked soils and porewater

Properties	NL1	NL2	NL3	NL4	NL5	NL6
Soil pH-$CaCl_2$	5.5	6.2	5.8	7.9	7.3	4.5
Total Cd (mg/kg)	0.65	0.17	0.36	0.13	0.64	0.28
Total Ni (mg/kg)	5.0	7.6	10.9	2.16	2.64	6.99
Organic matter (%)	6.4	5.7	21.7	10.2	12.3	2.0
CEC (cmol/kg)[a]	8.3	10.1	38.8	16.5	30.9	1.7
Texture	loamy sand	sandy loam	silt loam	clay loam	loam	sandy
Clay content (%)	5.9	7.0	16.5	33.8	27.1	0.8
Silt content (%)	8.9	22.5	53.2	46.1	40.9	2.9
Sand content (%)	85.2	70.5	30.3	20.1	32.0	96.3
Porewater pH	5.88	6.48	6.25	8.02	7.48	5.00
DOC (mg/L)[b]	149	182	503	192	342	189
Dissolved Na (mg/L)	41.3	66.1	119	97.4	45.0	43.8
Dissolved K (mg/L)	191	68.8	39.0	15.3	31.7	96.8
Dissolved Ca (mg/L)	120	199	509	370	337	101
Dissolved Mg (mg/L)	92.6	71.5	141	56.3	51.5	79.4

[a]Cation exchange capacity; [b]Dissolved organic carbon.

Table 3.2 Linear regression relationships between dissolved Cd concentrations in soil porewater (Cd_{pw}, mg/L), total soil Cd concentrations (Cd_{total}, mg/kg), and pH of the porewater in the six tested soils

Soils	Equations	R^2_{adj} [a]	n	F	p
NL1	$\log Cd_{pw} = 0.99\ (\pm0.11) \log Cd_{total} - 1.49\ (\pm0.28)$	0.89	11	76.3	<0.001
	$\log Cd_{pw} = 1.13\ (\pm0.06) \log Cd_{total} - 0.33\ (\pm0.07)\ pH + 0.11\ (\pm0.36)$	0.97	11	158.2	<0.001
NL2	$\log Cd_{pw} = 0.91\ (\pm0.08) \log Cd_{total} - 1.46\ (\pm0.23)$	0.89	15	110.3	<0.001
	$\log Cd_{pw} = 1.02\ (\pm0.03) \log Cd_{total} - 0.42\ (\pm0.04)\ pH + 1.04\ (\pm0.27)$	0.99	15	494.8	<0.001
NL3	$\log Cd_{pw} = 0.78\ (\pm0.05) \log Cd_{total} - 1.32\ (\pm0.17)$	0.92	16	183.7	<0.001
	$\log Cd_{pw} = 0.85\ (\pm0.06) \log Cd_{total} - 0.26\ (\pm0.12)\ pH + 0.11\ (\pm0.72)$	0.94	16	116.7	<0.001
NL4	$\log Cd_{pw} = 1.11\ (\pm0.14) \log Cd_{total} - 2.58\ (\pm0.41)$	0.83	14	62.9	<0.001
	$\log Cd_{pw} = 1.09\ (\pm0.04) \log Cd_{total} - 0.90\ (\pm0.08)\ pH + 4.29\ (\pm0.63)$	0.98	14	413.9	<0.001
NL5	$\log Cd_{pw} = 1.09\ (\pm0.16) \log Cd_{total} - 2.39\ (\pm0.49)$	0.78	14	42.6	<0.001
	$\log Cd_{pw} = 1.17\ (\pm0.11) \log Cd_{total} - 0.43\ (\pm0.10)\ pH + 0.41\ (\pm0.77)$	0.90	14	57.8	<0.001
NL6	$\log Cd_{pw} = 1.28\ (\pm0.24) \log Cd_{total} - 1.47\ (\pm0.43)$	0.85	7	28.3	<0.001
	$\log Cd_{pw} = 1.21\ (\pm0.04) \log Cd_{total} - 0.51\ (\pm0.05)\ pH + 1.25\ (\pm0.28)$	0.99	7	430.4	<0.001
All soils	$\log Cd_{pw} = 0.72\ (\pm0.07) \log Cd_{total} - 1.09\ (\pm0.19)$	0.59	77	106.4	<0.001
	$\log Cd_{pw} = 0.97\ (\pm0.04) \log Cd_{total} - 0.44\ (\pm0.03)\ pH + 1.09\ (\pm0.18)$	0.90	77	308.7	<0.001

[a] R^2_{adj} is the coefficient of determination adjusted for the degrees of freedom; n indicates the number of data points; F is the value of F test; p indicates the statistical significance level; Standard errors are indicated in brackets.

Table 3.3 Linear regression relationships between dissolved Ni concentrations in soil porewater (Ni_{pw}, mg/L), total soil Ni concentrations (Ni_{total}, mg/kg), and pH of the porewater in the six tested soils

Soils	Equations	R^2_{adj} [a]	*n*	*F*	*p*
NL1	$\log Ni_{pw} = 2.14\ (\pm 0.32) \log Ni_{total} - 4.36\ (\pm 0.86)$	0.87	8	42.9	<0.001
	$\log Ni_{pw} = 2.01\ (\pm 0.20) \log Ni_{total} - 1.02\ (\pm 0.34)\ pH + 2.49\ (\pm 2.34)$	0.95	8	60.4	<0.001
NL2	$\log Ni_{pw} = 1.34\ (\pm 0.16) \log Ni_{total} - 2.36\ (\pm 0.48)$	0.88	11	69.7	<0.001
	$\log Ni_{pw} = 1.37\ (\pm 0.12) \log Ni_{total} - 0.38\ (\pm 0.14)\ pH + 0.04\ (\pm 1.00)$	0.93	11	63.1	<0.001
NL3	$\log Ni_{pw} = 0.81\ (\pm 0.11) \log Ni_{total} - 1.19\ (\pm 0.36)$	0.82	14	56.3	<0.001
	$\log Ni_{pw} = 1.13\ (\pm 0.09) \log Ni_{total} - 0.36\ (\pm 0.08)\ pH + 0.01\ (\pm 0.34)$	0.94	14	88.4	<0.001
NL4	$\log Ni_{pw} = 0.91\ (\pm 0.11) \log Ni_{total} - 2.88\ (\pm 0.39)$	0.82	16	63.4	<0.001
	$\log Ni_{pw} = 0.83\ (\pm 0.08) \log Ni_{total} - 0.60\ (\pm 0.17)\ pH + 2.08\ (\pm 1.49)$	0.90	16	62.9	<0.001
NL5	$\log Ni_{pw} = 1.13\ (\pm 0.14) \log Ni_{total} - 2.65\ (\pm 0.48)$	0.86	12	67.3	<0.001
	$\log Ni_{pw} = 1.15\ (\pm 0.15) \log Ni_{total} - 0.03\ (\pm 0.15)\ pH - 2.95\ (\pm 1.38)$	0.85	12	30.1	<0.001
NL6	$\log Ni_{pw} = 2.17\ (\pm 0.20) \log Ni_{total} - 3.52\ (\pm 0.36)$	0.94	7	111.1	<0.001
	$\log Ni_{pw} = 2.33\ (\pm 0.14) \log Ni_{total} - 0.89\ (\pm 0.32)\ pH + 0.84\ (\pm 1.57)$	0.98	7	135.5	<0.001
All soils	$\log Ni_{pw} = 0.70\ (\pm 0.14) \log Ni_{total} - 1.15\ (\pm 0.43)$	0.29	68	26.2	<0.001
	$\log Ni_{pw} = 1.45\ (\pm 0.13) \log Ni_{total} - 0.84\ (\pm 0.10)\ pH + 2.27\ (\pm 0.52)$	0.66	68	60.6	<0.001

[a] R^2_{adj} is the coefficient of determination adjusted for the degrees of freedom; *n* indicates the number of data points; *F* is the value of *F* test; *p* indicates the statistical significance level; Standard errors are indicated in brackets.

Table 3.4 Total metal concentrations ([Cd] and [Ni], mg/kg), and free metal ion activity ({Cd^{2+}} and {Ni^{2+}}, M) causing 50% mortality of the earthworms *Lumbricus rubellus* and *Aporrectodea longa* (with the corresponding 95% confidence intervals in parentheses) exposed in different test soils for 28 d.

Soils	*L. rubellus*				*A. longa*			
	LC50[Cd] [a]	pLC50{Cd^{2+}} [b]	LC50[Ni]	pLC50{Ni^{2+}}	LC50[Cd]	pLC50{Cd^{2+}}	LC50[Ni]	pLC50{Ni^{2+}}
NL1	63	5.48	242	4.35	141	5.06	380	3.96
	(48-83)	(5.38-5.59)	(199-295)	(4.08-4.63)	(99-202)	(4.77-5.35)	(285-506)	(3.63-4.28)
NL2	127	5.16	557	3.77	212	4.87	1294	3.63
	(84-192)	(4.92-5.40)	(497-624)	(3.69-3.85)	(181-248)	(4.78-4.96)	(1044-1605)	(3.38-3.88)
NL3	339	5.34	2354	3.71	1231	4.73	4039	3.38
	(232-494)	(5.15-5.52)	(1715-3232)	(3.57-3.85)	(954-1588)	(4.63-4.82)	(3345-4877)	(3.34-3.42)
NL4	410	5.43	3470	4.26	1409	4.80	5266	3.81
	(327-514)	(5.23-5.64)	(3001-4012)	(4.18-4.34)	(1167-1700)	(4.73-4.88)	(4577-6059)	(3.69-3.93)
NL5	319	5.57	2163	4.01	900	4.91	4253	3.65
	(241-421)	(5.35-5.79)	(1889-2476)	(3.88-4.14)	(747-1085)	(4.84-4.98)	(3739-4839)	(3.63-3.68)
NL6	37	5.25	70	4.58	49	5.10	139	4.07
	(31-44)	(5.10-5.40)	(54-90)	(4.16-5.00)	(40-59)	(4.87-5.33)	(105-185)	(3.98-4.16)

[a]LC50 indicates median lethal concentration; [b]pLC50 = −logLC50

In order to tentatively identify the possible protective effects of other cations (H^+, Ca^{2+}, Mg^{2+}, Na^+ and K^+) the effective dose term, consisting of an extra cation as well as Cd^{2+}, was used to fit the data with equation 3-5. The increase in R^2 and decrease in RMSE by using different effective dose terms compared with the model using $\{Cd^{2+}\}$ as the sole descriptor were marginal and the Akaike information criterion favored the latter for both earthworm species tested (Supporting Information, Table S3.3). No significant competing effects of other cations with Cd^{2+} for the biotic ligands were observed.

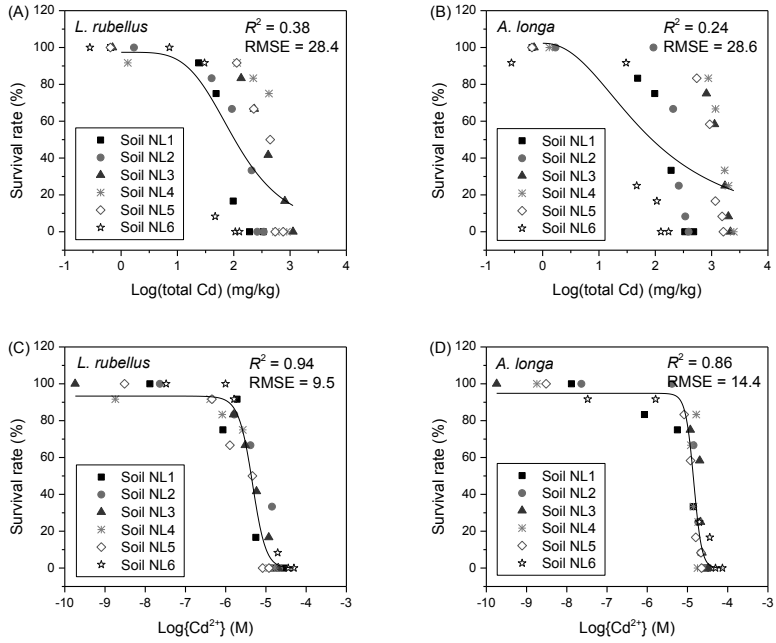

Figure 3.1 Dose-response relationships between survival rate and total Cd concentration in soil (first row), free Cd^{2+} activity in soil porewater (second row) for the earthworms *Lumbricus rubellus* and *Aporrectodea longa* exposed to 6 soils for 28 d. The solid lines represent the logistic model fits. R^2 indicates the determination coefficient of the linear regression between the predicted and observed survival rate. RMSE indicates the root-mean-square error of the predicted survival rate versus the observed values.

Dose−response relationships for Ni toxicity

The relationships between the survival of earthworms and different expressions of exposure of Ni are shown in Figure 3.2. Increasing total Ni concentrations decreased the survival of earthworms. Up to 50- and 38-fold differences in Ni toxicity (LC50[Ni]) occurred for *L. rubellus* and *A. longa*, respectively (Table 3.4). In all soils tested, *L. rubellus* was more sensitive to Ni than *A. longa*. When using total Ni concentration as the expression for Ni toxicity across different soils, poor fits were obtained with R^2 of 0.26 and RMSE of 33.4 for *L. rubellus*, and R^2 of 0.30 and RMSE of 29.4 for *A. longa* (Figure 3.2A and 3.2B). The model fits were considerably improved with $\{Ni^{2+}\}$ as the descriptor for toxicity (Figure 3.2C

and 3.2D). The R^2 and RMSE values were 0.77 and 19.7 for *L. rubellus*, and 0.78 and 16.9 for *A. longa*, respectively. When based on $\{Ni^{2+}\}$ in soil porewater, differences in LC50$\{Ni^{2+}\}$ for the different soils were smaller (a variation of 7.4-fold for *L. rubellus* and 4.9-fold for *A. longa*) (Table 3.4).

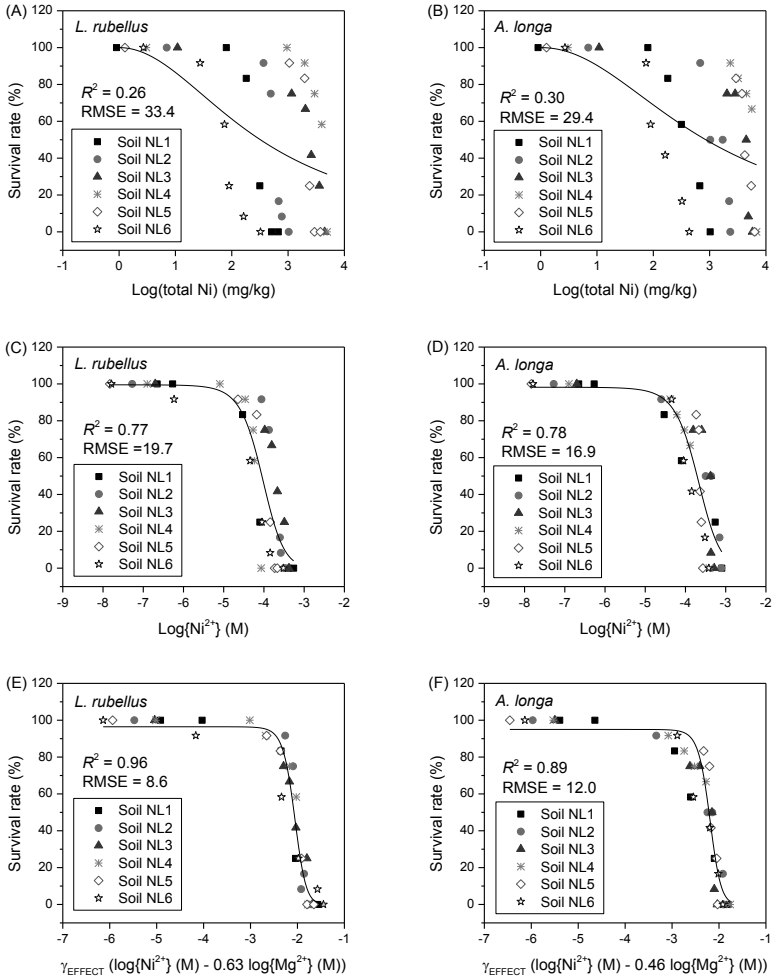

Figure 3.2 Dose-response relationships between the 28-d survival rate of earthworms *Lumbricus rubellus* and *Aporrectodea longa*, and different expressions of Ni exposure in 6 soils (soil total concentration, free Ni^{2+} activity in soil porewater, and effective dose (equation 3-2). The solid lines represent the logistic model fits. R^2 indicates the determination coefficient of the linear regression between the predicted and observed survival rate. RMSE indicates the root-mean-square error of the predicted survival rate versus the observed values.

To gain further insight into the importance of other cations in modifying Ni toxicity, the effective dose term comprising $\{Ni^{2+}\}$ and one of the variables (H^+, Ca^{2+}, Mg^{2+}, Na^+, and K^+)

was substituted into the model (equation 3-5) to fit the entire data set. Adding Mg^{2+} into the model gave the best prediction (R^2 = 0.96 and RMSE = 8.6 for *L. rubellus* (Figure 3.2E); R^2 = 0.89 and RMSE = 12.0 for *A. longa* (Figure 3.2F)) and addition of the other cations slightly improved the model fits (Supporting Information, Table S3.4). The effective dose term was further extended to include Ni^{2+}, Mg^{2+} and one of the other cations (H^+, Ca^{2+}, Na^+, and K^+). There were only marginal improvements in model performance (Supporting Information, Table S3.4). The AIC favored the effective dose terms:

$$\gamma_{effect} = \log\{Ni^{2+}\} - 0.63 \log\{Mg^{2+}\} \text{ for } L.\ rubellus \quad (3\text{-}6)$$

$$\gamma_{effect} = \log\{Ni^{2+}\} - 0.46 \log\{Mg^{2+}\} \text{ for } A.\ longa \quad (3\text{-}7)$$

Thus, Mg^{2+} was found to be a dominant factor in modifying Ni^{2+} toxicity to the two earthworm species, while the role of H^+, Ca^{2+}, Na^+, and K^+ was not appreciable.

Predictions of LC50s

An overall LC50{Cd^{2+}} was obtained by applying the FIAM to the toxicity data for each earthworm species in all soils together. This FIAM predicted that LC50{Cd^{2+}} never differed by a factor of more than 2 from the observed LC50{Cd^{2+}} (Figure 3.3A), indicating that {Cd^{2+}} sufficiently explained variations in Cd toxicity among soils. Thus, there was no further need to develop other predictive models for Cd toxicity.

Similarly, an overall LC50{Ni^{2+}} was obtained for each species in all soils together. The FIAM predicted that LC50{Ni^{2+}} was more than a factor of 2 of the observed values in some cases (3 out of 6 values for *L. rubellus*, 2 out of 6 values for *A. longa*) (Figure 3.3B). As it was not possible to predict the LC50{Ni^{2+}} in all cases within an error factor of 2 using the FIAM, the free ion approach was used to directly link toxicity to the porewater composition. Using the interpolated soil porewater properties corresponding to the total soil metal concentration at LC50[Ni] (Supporting Information, Table S3.5-S3.6) as input parameters, the activities of other cations (H^+, Ca^{2+}, Mg^{2+}, Na^+ and K^+) in soil porewater were then calculated with WHAM VI. The coefficient for each porewater parameter in equation 3-1 was estimated by stepwise multiple linear regression with the observed LC50{Ni^{2+}} as the dependent variable and the calculated activities of other cations as independent variables, yielding:

For *L. rubellus*:

$$\text{Log LC50}\{Ni^{2+}\} = 1.18\ (\pm 0.19) \log\{Mg^{2+}\} - 0.52\ (\pm 0.15)$$

$$(R^2_{adj} = 0.89,\ n = 6,\ p < 0.01,\ F = 36.6) \quad (3\text{-}8)$$

For *A. longa*:

$$\text{Log LC50}\{Ni^{2+}\} = 0.51\ (\pm 0.11) \log\{Mg^{2+}\} - 2.16\ (\pm 0.34)$$

$$(R^2_{adj} = 0.84,\ n = 6,\ p < 0.01,\ F = 21.5) \quad (3\text{-}9)$$

Only Mg^{2+} was identified as the explanatory variable and incorporated into the equations for predicting LC50{Ni^{2+}}. Other cations were the excluded variables as they did not pass stepping criteria. To verify the model parameters, LC50{Ni^{2+}} was predicted by filling in the estimated model parameters in the above equations. The resulting values were then compared with the experimentally measured values. Figure 3.3C shows the accuracy of model predictions. Predicted LC50{Ni^{2+}} were in good agreement with the observed values, with an error of far less than a factor of 2.

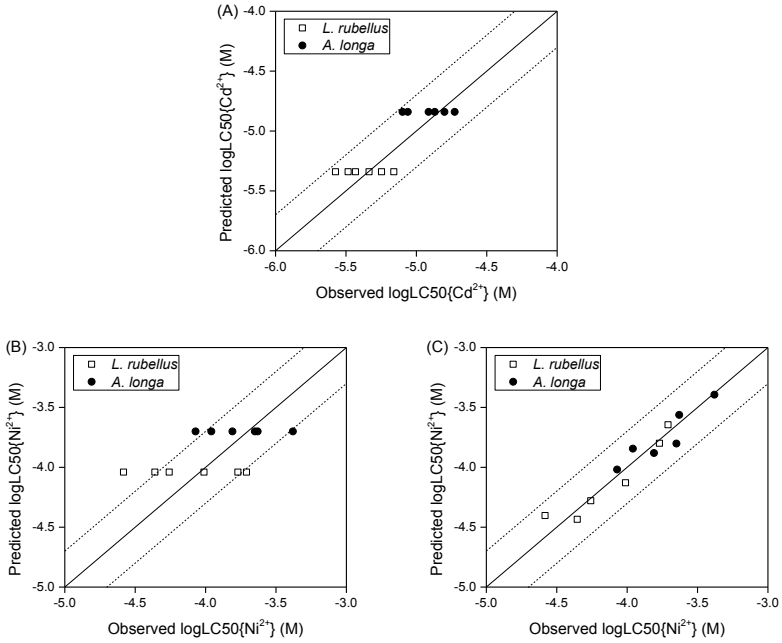

Figure 3.3 Relationship between the predicted and observed logLC50 expressed as free Cd^{2+} or Ni^{2+} activity in soil porewater for the earthworms *Lumbricus rubellus* and *Aporrectodea longa* in all 6 tested soils after 28 d of exposure. The predictions for logLC50{Cd^{2+}} were based on the free ion activity model (A). The predictions for logLC50{Ni^{2+}} were based on the free ion activity model (B) and the free ion approach (C) using the equations 8 and 9. The solid line represents the 1:1 line, and the dashed lines are a factor of 2 above and below the 1:1 line.

3.4 Discussion

A wide variation of Cd and Ni toxicity to earthworms among different soils stressed the importance of taking bioavailability and toxicity-modifying factors into account. In developing models for predicting metal toxicity to earthworms, dermal uptake of free metal ions present in soil porewater is assumed as the main route of exposure (Steenbergen et al., 2005; Vijver et al., 2003). According to the basic assumption of the BLM, solution cations (e.g., H^+, Ca^{2+}, Mg^{2+}, Na^+, and K^+) may alleviate metal toxicity through site-specific competition with metals for binding to the same active sites (transport sites or ion channels) of the organisms (De Schamphelaere and Janssen, 2002). Previously, it has been reported that all the above-mentioned cations alleviated Cd toxicity to *E. fetida* (Li et al., 2008), and that adding Mg^{2+} reduced Ni toxicity to plants (Lock et al., 2007). Therefore, in the present study, these cations in soil porewater were selected to determine if they exert a significant protective role on Cd and Ni toxicity to the two earthworm species tested.

Cd toxicity predictions

In the present study, $\{Cd^{2+}\}$ sufficiently explained variations in Cd toxicity across soils. There were no significant improvements in the predictive ability of the model by accounting for competition of other cations (H^+, Ca^{2+}, Mg^{2+}, Na^+ and K^+). This finding provided direct evidence that the organisms respond to free Cd^{2+}. It was expected that Ca^{2+} would alleviate Cd^{2+} toxicity according to the hypothesis that Ca^{2+} and Cd^{2+} compete for the same binding sites (transport sites) (Niyogi and Wood, 2004). However, no significant effect of Ca^{2+} on Cd^{2+} toxicity was observed in the present study. This suggests that Ca^{2+} was not necessary to disrupt Cd^{2+} uptake by earthworms. The lack of improvement upon correction for Ca^{2+} might be attributed to the fact that Ca^{2+} positively or negatively correlated with other porewater parameters (see Supporting Information, Table S3.1). Besides, it should be noted that differences in porewater cation concentrations between the tested soils were generally small (approximately a factor of 5 for dissolved Ca concentrations), which may hinder the cations from manifesting their protective role.

Many publications have shown that the overall ability of the free metal ion to reflect toxicity of metals for aquatic and terrestrial organisms is limited (De Schamphelaere and Janssen, 2002; Steenbergen et al., 2005; Van Gestel and Koolhaas, 2004). The FIAM is a special case of the free ion approach with no impact of cations. It is remarkable that the overall LC50$\{Cd^{2+}\}$ predicted with FIAM was within a factor of 2 of the individual LC50$\{Cd^{2+}\}$ for the two earthworm species in such diverse soils. The presence of protons and other cations reacting with biological binding sites was supposed to affect Cd toxicity according to the BLM theory. A Cd-BLM was previously developed for the earthworm *E. fetida* exposed in a hydroponic system (Li et al., 2008). Competing effects of H^+, Ca^{2+}, Mg^{2+}, Na^+ and K^+ were observed and all integrated into the developed BLM. There was up to 1.7-fold difference between the predicted and observed LC50$\{Cd^{2+}\}$ even though the difference in the observed LC50$\{Cd^{2+}\}$ was always less than a factor of 2.2 for all cation sets. It therefore seems reasonably to use $\{Cd^{2+}\}$ only to reflect the toxicity of Cd without considering the presence of protons and other cations. Ardestani et al. (2013) studied the impact of Ca and pH on the toxicity of Cd to *Folsomia candida* exposed to a simplified soil solution. They found that increasing Ca^{2+} activities had inconsistent effects on Cd toxicity and that toxicity was slightly lower at pH 7 and 6 than at pH 5. This finding does not seem to be in agreement with the BLM assumptions of cation (proton) competition, which should alleviate metal toxicity.

Ni toxicity predictions

A mitigating effect was shown for Mg^{2+} on Ni^{2+} toxicity to both *L. rubellus* and *A. longa*. The interaction of Mg^{2+} and Ni^{2+} has been widely reported (Lock et al., 2007; Thakali et al., 2006b; Antunes and Kreager, 2009). Similar ionic radii are found in Mg^{2+} and Ni^{2+} (0.066 nm for Mg^{2+} and 0.069 nm for Ni^{2+}), which enables Mg^{2+} to compete with Ni^{2+} for the Mg^{2+} channel or with carriers (Antunes and Kreager, 2009). Except for Mg^{2+}, the protective effects of H^+, Ca^{2+}, Na^+, and K^+ on Ni^{2+} toxicity to earthworms were negligible. Actually, when compared with the model using $\{Ni^{2+}\}$ solely as the variable, the addition of Ca^{2+} or Na^+ did add some value to model performance, but this might be a result of the significant correlation between dissolved Ca and Mg concentrations (r = 0.74), and between dissolved Na and Mg

concentrations (r = 0.81) (Supporting Information, Table S3.2). In addition to Ni^{2+} and Mg^{2+}, the contribution of an extra parameter (Ca^{2+} or Na^{+}) to accuracy of model prediction was marginal as indicated by the AIC values. This led to the conclusion that only Mg^{2+} significantly alleviated Ni^{2+} toxicity. In practice, the concentration of major cations in soil porewater often tends to be negatively correlated with the porewater pH (Wang et al., 2011a). Therefore, porewater pH was expected to exert a significant role in modifying metal toxicity as it can not only represent the role of H^{+}, but also the overall role of other cations (Lofts et al., 2004). The lack of protective effects of pH in the present study might be a result of the significant protective effects of Mg^{2+}; that is, Mg^{2+} suppressed or masked the competitive effect of H^{+}.

A lack of consistent effects from other cations was found in the literature. The alleviative effects of H^{+}, Ca^{2+} and Mg^{2+} were found for Ni toxicity to *Hordeum vulgare* root elongation and *Lycopersicon esculentum* shoot yield. Both H^{+} and Mg^{2+} competitions were observed for *E. fetida* cocoon production and *F. candida* reproduction (Thakali et al., 2006b). Only Mg^{2+} had an effect on Ni toxicity to *H. vulgare* root elongation (Lock et al., 2007). This might be attributed to several causes, such as covariance between H^{+} and other cations, species-specific ion binding ability, the use of different endpoints and differences in exposure medium.

The interaction of Mg^{2+} with Ni^{2+} for the biotic ligands, as considered by the free ion approach, accurately predicted the LC50$\{Ni^{2+}\}$. This shows the applicability of the free ion approach for predicting Ni toxicity to earthworms in a range of soils. The coefficients for $\log\{Mg^{2+}\}$ (i.e., n_{Mg}/n_{Ni}) in equations 3-8 and 3-9 quantified the competition of Mg^{2+} with Ni^{2+} at the biotic ligands. These values were different for the two earthworm species, suggesting that the protective effects of Mg^{2+} on Ni^{2+} toxicity were species-dependent. In applying BLM theory to the soil system, parameterization of the BLM faces a variety of challenges and uncertainties (Erickson, 2013; Thakali et al., 2006b). The free ion approach avoids the need to fit separate affinity parameters for the toxic metal and potentially competing ions as the coefficients for each cation express the binding affinities of protective cations relative to metal ions (Lofts et al., 2013; Qiu et al., 2013). When developing a predictive model, it would be highly desirable to make the model as simple as possible, and to avoid over-parameterization as that suggests details on processes that cannot be justified (Van Zelm and Huijbregts, 2013). The ultimate goal for modellers should be, to meet the law of parsimony (Occam's Razor), to look for simple explanation of the observed phenomena rather postulating complex processes without empirical evidence (Hauschild et al., 2008). The free ion approach, therefore, is the preferred one when compared with the BLM or the complicated electrostatic model (Wang et al., 2011b; 2013) in predicting soil metal toxicity.

In the present study, Cd toxicity to earthworms was found to be driven by the $\{Cd^{2+}\}$ alone. In case of Ni, alleviation of Ni^{2+} toxicity by Mg^{2+} was observed. Previously, a pH dependence of Cu^{2+} toxicity to different earthworm species was reported (Qiu et al., 2013). These findings clearly showed that there is a lack of consistent effects from the presence of possible competing cations for different metals. Therefore, care should be taken when extrapolating the free ion approach across metals. We therefore strongly suggest that metal toxicity to earthworms needs to be evaluated on a metal-specific basis.

3.5 Conclusions

The free ion activity was a sufficient descriptor for Cd toxicity across the different soils, while it was in itself unsuccessful in describing the variability in Ni toxicity. The free ion approach, in line with the basic assumptions of the BLM, seemed to be a feasible way of modelling both Cd and Ni toxicity to different earthworms. As validity is proven, it may replace BLM or other complicated approaches and increase the applicability for site-specific risk assessment.

Supporting Information

Table S3.1 Pearson's correlation between porewater parameters (pH, dissolved Ca, Mg, Na, K, and Cd) for all unspiked and Cd-spiked soils

	Range	Correlation						
		pH	DOC	Ca	Mg	Na	K	Cd
pH	4.5-7.9	1	-0.11	0.39**	-0.27*	0.17	-0.61**	-0.01
DOC	92-569 mg/L		1	0.42**	0.42**	0.30*	-0.20	0.21
Ca	110-572 mg/L			1	0.60**	0.72**	-0.47**	-0.12
Mg	33-182 mg/L				1	0.68**	0.21	0.06
Na	28-136 mg/L					1	-0.19	0.03
K	11-229 mg/L						1	-0.10
Cd	0-31 mg/L							1

**Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

Table S3.2 Pearson's correlation between porewater parameters (pH, dissolved Ca, Mg, Na, K, and Ni) for all unspiked and Ni-spiked soils

	Range	Correlation						
		pH	DOC	Ca	Mg	Na	K	Ni
pH	4.6-7.9	1	-0.12	0.15	-0.27*	0.00	-0.39**	-0.07
DOC	95-561 mg/L		1	0.56**	0.39**	0.24	-0.24	-0.04
Ca	57-600 mg/L			1	0.74**	0.79**	-0.17	0.04
Mg	13-186 mg/L				1	0.81**	0.44**	0.29*
Na	14-133 mg/L					1	0.14	0.15
K	10-157 mg/L						1	0.29*
Ni	0-132 mg/L							1

**Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

Table S3.3 Fitting statistics for models (equation 5) using different effective dose terms for predicting the effect of Cd on the survival of earthworms *Lumbricus rubellus* and *Aporrectodea longa* in all six tested soils after 28 days of exposure. The parameters were estimated by multiple nonlinear regression (n = 36).

species	Effective dose	Model fit		
		R^2_{adj} [a]	RMSE [b]	AIC [c]
L. rubellus	$\log\{Cd^{2+}\}$ [d]	0.943	9.5	170
	$\log\{Cd^{2+}\}$ - 0.02 pH	0.947	9.5	172
	$\log\{Cd^{2+}\}$ + 0.008 $\log\{Ca^{2+}\}$	0.946	9.5	172
	$\log\{Cd^{2+}\}$ + 0.16 $\log\{Mg^{2+}\}$	0.947	9.5	172
	$\log\{Cd^{2+}\}$ - 0.14 $\log\{Na^{+}\}$	0.947	9.4	172
	$\log\{Cd^{2+}\}$ + 0.07 $\log\{K^{+}\}$	0.947	9.5	172
A. longa	$\log\{Cd^{2+}\}$ [d]	0.862	14.4	200
	$\log\{Cd^{2+}\}$ + 0.006 pH	0.862	14.3	202
	$\log\{Cd^{2+}\}$- 0.232 $\log\{Ca^{2+}\}$	0.870	13.9	200
	$\log\{Cd^{2+}\}$ - 0.102 $\log\{Mg^{2+}\}$	0.864	14.3	201
	$\log\{Cd^{2+}\}$ - 0.214 $\log\{Na^{+}\}$	0.871	13.9	200
	$\log\{Cd^{2+}\}$ - 0.004 $\log\{K^{+}\}$	0.862	14.4	202

[a] R^2_{adj} is the explained variance; [b] RMSE = Root mean squared error; [c] Akaike information criterion; [d] Model favoured.

Table S3.4 Fitting statistics for models (equation 5) using different effective dose terms for predicting the effect of Ni on the survival of earthworms *Lumbricus rubellus* and *Aporrectodea longa* in all six tested soils after 28 days of exposure. The parameters were estimated by multiple-nonlinear-regression (n = 36).

Species	Effective dose term	Model fit		
		R^2_{adj} [a]	RMSE [b]	AIC [c]
L.rubellus	$\log\{Ni^{2+}\}$	0.768	19.4	221
	$\log\{Ni^{2+}\}$ - 0.628 $\log\{Mg^{2+}\}$ [d]	0.955	8.6	164
	$\log\{Ni^{2+}\}$ - 0.001 pH	0.768	19.4	223
	$\log\{Ni^{2+}\}$ - 0.504 $\log\{Ca^{2+}\}$	0.862	15.0	205
	$\log\{Ni^{2+}\}$ - 0.943 $\log\{Na^{+}\}$	0.867	14.7	203
	$\log\{Ni^{2+}\}$ - 0.219 $\log\{K^{+}\}$	0.785	18.7	221
	$\log\{Ni^{2+}\}$ - 0.723 $\log\{Mg^{2+}\}$ - 0.083 pH	0.960	8.0	162
	$\log\{Ni^{2+}\}$ - 0.515 $\log\{Mg^{2+}\}$ - 0.180 $\log\{Ca^{2+}\}$	0.959	8.2	163
	$\log\{Ni^{2+}\}$ - 0.513 $\log\{Mg^{2+}\}$ - 0.333 $\log\{Na^{+}\}$	0.956	8.4	166
	$\log\{Ni^{2+}\}$ - 0.712 $\log\{Mg^{2+}\}$ + 0.114 $\log\{K^{+}\}$	0.957	8.3	165
A. longa	$\log\{Ni^{2+}\}$	0.784	16.9	212
	$\log\{Ni^{2+}\}$ - 0.456 $\log\{Mg^{2+}\}$ [d]	0.892	12.0	189
	$\log\{Ni^{2+}\}$ - 0.02 pH	0.785	16.9	214
	$\log\{Ni^{2+}\}$ - 0.462 $\log\{Ca^{2+}\}$	0.839	14.4	202
	$\log\{Ni^{2+}\}$ - 0.426 $\log\{Na^{+}\}$	0.821	15.5	207
	$\log\{Ni^{2+}\}$ - 0.136 $\log\{K^{+}\}$	0.791	16.7	213
	$\log\{Ni^{2+}\}$ - 0.455 $\log\{Mg^{2+}\}$ - 0.013 pH	0.893	11.9	191
	$\log\{Ni^{2+}\}$ - 0.305 $\log\{Mg^{2+}\}$ - 0.240 $\log\{Ca^{2+}\}$	0.902	11.5	188
	$\log\{Ni^{2+}\}$ - 0.524 $\log\{Mg^{2+}\}$ + 0.116 $\log\{Na^{+}\}$	0.897	11.7	189
	$\log\{Ni^{2+}\}$ - 0.511 $\log\{Mg^{2+}\}$ + 0.081 $\log\{K^{+}\}$	0.893	12.0	191

[a] R^2_{adj} is the explained variance; [b] RMSE = Root mean squared error; [c] Akaike information criterion; [d] Model favoured.

Table S3.5 The soil porewater properties (pH, dissolved organic carbon (DOC), and dissolved cations concentrations) interpolated at LC50 values of Ni (expressed as total soil concentration) for *Lumbricus rubellus* in the six soils tested. The function TREND in Microsoft Excel 2010 was used for interpolation based on the measured values at the tested exposure concentrations.

Soil	LC50 of Ni (mg kg^{-1})	Porewater pH	DOC (mg L^{-1})	Dissolved cations in soil porewater (mg L^{-1})				
				Ca	Mg	Na	K	Ni
NL1	242	6.42	244	126	86.6	57.5	201	18.8
NL2	557	6.52	158	232	88.0	78.4	99.0	25.0
NL3	2354	6.26	445	496	138	105	39.1	34.7
NL4	3470	7.89	198	204	24.9	64.2	8.70	24.0
NL5	2163	7.45	349	280	44.1	30.4	26.8	18.8
NL6	70	5.14	221	83.0	47.6	42.1	106	6.35

Table S3.6 The soil porewater properties (pH, dissolved organic carbon (DOC), and dissolved cations concentrations) interpolated at LC50 values of Ni (expressed as total soil concentration) for *Aporrectodea longa* in the six soils tested. The function TREND in Microsoft Excel 2010 was used for interpolation based on the measured values at the tested exposure concentrations.

Soil	LC50 of Ni (mg kg^{-1})	Porewater pH	DOC (mg L^{-1})	Dissolved cations in soil porewater (mg L^{-1})				
				Ca	Mg	Na	K	Ni
NL1	380	6.42	235	130	93.1	59.0	206	34.5
NL2	1294	6.49	167	251	99.3	85.5	126	68.7
NL3	3046	6.37	479	507	135	109	38.8	45.7
NL4	5266	7.62	248	201	21.6	65.1	8.46	33.2
NL5	4253	7.42	363	217	29.0	25.6	19.8	33.9
NL6	139	5.04	225	70.9	40.6	39.2	94.5	15.8

Chapter IV

Can Commonly Measurable Traits Explain Differences In Metal Accumulation And Toxicity In Earthworm Species?

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Abstract

There is no clear consensus in the literature on the metal accumulation pattern and sensitivity of different earthworm species. In the present study, accumulation and toxicity of Cu, Cd, Ni, and Zn in the earthworms *Lumbricus rubellus* (epigeic), *Aporrectodea longa* (anecic), and *Eisenia fetida* (ultra-epigeic) were determined after 28 days exposure in two soils. Metal accumulation and sensitivity were interpreted using the specific traits of different earthworm species. Results showed that for all four metals tested *L. rubellus* was the most sensitive species, followed by *A. longa* and *E. fetida*. At the same exposure concentration, internal concentrations followed the order: *L. rubellus* > *E. fetida* > *A. longa* for Cu and Ni, *L. rubellus* ≈ *E. fetida* ≈ *A. longa* for Cd, and *L. rubellus* > *A. longa* > *E. fetida* for Zn. Langmuir isotherms were used to model metal accumulation at both nontoxic and toxic exposure concentrations. The Cu, Cd, and Zn concentrations in *E. fetida* generally leveled off at high exposure concentrations but not for the other two species. *A. longa* showed a high capability of regulating internal Ni concentrations. The traits-based approaches suggested that most likely a group of earthworm traits together determined (differences in) metal accumulation and sensitivity. More research is needed in this respect to build up solid relationships between species-specific responses and traits, enabling cross-species extrapolation of accumulation and toxicity data.

4.1 Introduction

Metal contamination in the topsoil usually results from anthropogenic activities such as waste disposal, agricultural inputs, and industrial emissions (Onianwa et al., 2003). Adverse effects of elevated concentrations of metals (e.g., Cu, Cd, Ni, and Zn) on soil functions and on soil-dwelling organisms have been widely recognized (Lock and Janssen, 2001; Santorufo et al., 2012). Earthworms are ecosystem engineers which play a unique role in soil formation and soil fertility (Blouin et al., 2013). They are especially active in the topsoil and continuously exposed to metals through direct contact with soil particles and porewater, providing an early indication of metal pollution and ecosystem disturbance (Nahmani et al., 2007; Sizmur and Hodson, 2009; Spurgeon et al., 2006).

Earthworms are generally classified into three ecotypes according to their ecological strategies: epigeics, endogeics and anecics (Bouché, 1977). The epigeic earthworms (such as *Lumbricus rubellus* and *Dendrobaena octaedra*) live on the surface of the soil in leaf litter. They feed on the surface litter and are found intimately associated with plant roots (Curry and Schmidt, 2007; Domínguez, 2004). The endogeic earthworms (such as *Aporrectodea caliginosa* and *Allolobophora chlorotica*) are mineral soil-dwelling species. They feed predominantly within the mineral soil and rarely venture to the soil surface (Bouché, 1977; Curry and Schmidt, 2007). The anecic earthworms (such as *Aporrectodea longa* and *Lumbricus terrestris*) make permanent vertical burrows in soil. They draw organic matter from the soil surface into their deep, permanent burrows to feed on (Butt, 1993; Domínguez, 2004). The above mentioned earthworm species are commonly found throughout Europe and have been introduced to North America (Hendrix and Bohlen, 2002). The ultra epigeic *Eisenia fetida*, currently used as the standard earthworm in terrestrial ecotoxicology testing (Langdon et al., 2005; Peijnenburg and Vijver, 2009), is cultured for vermicomposting or fishing and is rarely found in natural soils. It inhabits only organic matter-rich locations (e.g., composts and dung heaps) (Edwards and Arancon, 2004). These differences in ecological strategies have consequences for exposure, accumulation and sensitivity to metals. For instance, earthworm traits such as feeding behavior or the soil habitat (e.g., depth at which the earthworm species live) can affect the degree of contact with contaminated soil particles and therefore may cause differences in exposure to metals.

Earthworms have developed physiological mechanisms to adapt to metal exposure. They are able to actively regulate metal bioaccumulation via exclusion or increased elimination (Hopkin, 1989). Spurgeon and Hopkin (1999) showed that equilibrium body concentrations of Cu and Zn in *E. fetida* were reached after fast initial uptake, highlighting a physiological control and an effective excretion of these metals. For Cd and Pb, the excretion was slow or absent. Earthworms have the ability to prevent themselves from toxic effects by sequestration, detoxification, and storage of excess metal (Vijver et al., 2004), resulting in high accumulation levels. Ecophysiological studies showed that metal binding to proteins such as metallothioneins or sequestration in granules (Spurgeon and Hopkin, 1999; Vijver et al., 2004) occurs depending on metal species and earthworm types.

Metal accumulation varied largely between different earthworm species and *E. fetida* is often found to be less sensitive to metals than other species (Nahmani et al., 2007; Spurgeon et al., 2000). Many researchers believe that species-specific differences in ecological strategies and physiological characteristics (detoxification and elimination strategies) might

account for the differences in earthworm metal accumulation and sensitivity (Nahmani et al., 2007; Spurgeon et al., 2000; Spurgeon and Hopkin, 1996). In contrast, relatively little variation in metal accumulation between species was also reported (Langdon et al., 2005; Nannoni et al., 2011), although large differences in exposure concentrations were tested.

Based on the existing literature data, it was hypothesized that ecophysiological differences between earthworms will influence metal accumulation patterns and sensitivity, and that species-specific characteristics of earthworms will provide a clue for extrapolation across species. The main objectives of the present study were therefore as follows: (i) to determine the relative sensitivity of three different earthworm species (*L. rubellus, A. longa, and E. fetida*) to Cu, Cd, Ni, and Zn, (ii) to compare differences in accumulation patterns of each metal across different earthworm species, and (iii) to examine whether it is possible to extrapolate ecotoxicological effects from one earthworm species to another species using a traits-based approach.

4.2 Materials and methods

Earthworms

Mature earthworms *L. rubellus* and *A. longa* were collected from an unexploited site (nature reserve) in Leiden, the Netherlands. *E. fetida* was ordered from "Regenwormen Cultivation Farm" in Amsterdam, the Netherlands. Earthworms were kept in a climate room (15 °C, 16 h light and 8 h dark) and fed twice a week with organic-rich food (cow manure). All individuals of *L. rubellus*, *A. longa* and *E. fetida* selected for toxicity tests were adults with a well-developed clitellum. Their fresh weight ranged from 700 to 900 mg, 800 to 1000 mg, and 600 to 800 mg, respectively.

Test soils

Topsoil samples (0-15 cm) were collected at two agricultural sites (soil I and soil II) in the Netherlands. The soils were sieved (< 2 mm) and homogenised before use. Soil pH, organic matter content (OM), texture, cation exchange capacity (CEC) and maximum water-holding capacity were determined following the standard procedures described by Pansu and Gautheyrou (2006). Soil samples were spiked with Cd, Cu, Ni, and Zn in the form of metal acetate (Acros Chemicals; purity 98%). The designed levels of metal concentrations were soil- and metal-specific according to the previous studies (Spurgeon et al., 2000; Qiu et al., 2013). In soil I, except a control there were 9 levels of Cu concentrations (from 50 to 2000 mg/kg), 12 levels of Cd concentrations (from 50 to 3000 mg/kg), 10 levels of Ni concentrations (from 400 to 2800 mg/kg), and 13 levels of Zn concentrations (from 600 to 7000 mg/kg). In soil II, except a control there were 14 levels of Cu concentrations (from 50 to 4000 mg/kg), 14 levels of Cd concentrations (from 100 to 4500 mg/kg), 13 levels of Ni concentrations (from 800 to 7200 mg/kg), and 14 levels of Zn concentrations (from 800 to 10000 mg/kg). After spiking, the soils were subjected to alternated cycles of wetting and drying at 35 °C for two months to enhance acetate mineralization (Qiu et al., 2013).

Toxicity tests

Exposures were performed under controlled conditions at 15 °C for 28 days. Four randomly chosen earthworms were rinsed and put into plastic jars filled with 500 g soil samples. For each treatment, three replicates were used. All soils were maintained at a moisture content equivalent with 80% of the maximum water holding capacity during the experiment. During exposure, the earthworms were fed with cow manure (5g per jar) once a week. After 28 days of exposure, mortality was checked as it is the most frequently used endpoint (also scheduled in OECD guideline) to evaluate metal toxicity in earthworms (Van Gestel and Van Dis, 1988; Peijnenburg et al., 2009). Fresh body weight was measured before and at the end of the experiments. In the control soils, no significant loss of weight was observed after 28 days of exposure. The surviving earthworms were collected, washed and put on wetted filter paper to empty their guts for 48 hours. After gut voiding, earthworms were rinsed again and stored at minus 18 °C for further analysis.

Metal analysis

Soil samples were air-dried and sieved (< 2 mm). Earthworms were freeze dried for 48 hours. The soil and earthworm samples were digested using 1:4 v/v HCl (37% pro analysis, Baker) and HNO_3 (65% pro analysis; Riedel-de-Haen). After digestion, concentrations of Cu, Cd, Ni, and Zn in solution were analyzed by flame atomic absorption spectrophotometry (FAAS, Perkin Elmer AAnalyst 100). The certified reference materials ISE 989 and NRC DOLT-2 were used for analytical quality control. Measured concentrations were within 15% of the certified reference values.

Data analysis and statistics

Assuming that metal concentrations in earthworms would reach saturation at high exposure concentrations (Borgmann et al., 2004; Van Gestel et al., 2011), the relationship between metal concentrations in earthworms and total metal concentrations in soils were fitted by applying a Langmuir isotherm, which reads:

$$C_{\text{internal}} = \frac{C_{\max} \cdot K_L \cdot C_{\text{soil}}}{1 + K_L \cdot C_{\text{soil}}} \quad (4\text{-}1)$$

where C_{internal} is the metal concentration in earthworms (mg/kg dry body weight); $C_{\max}$ is the maximum metal concentration in earthworms (mg/kg dry body weight); C_{soil} is the total metal concentration in soil (mg/kg dry soil); K_L is the Langmuir constant (kg/mg). The Langmuir model was used to model metal uptake at all exposure concentrations (both nontoxic and toxic) except where 100% mortality occurred. Parameters estimation was performed by nonlinear regression using Origin 9.0 (Origin Lab, UK).

The metal concentrations causing 50% mortality (LC50) and their 95% confidence intervals were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). All the data were statistically analyzed using one way analysis of variance (ANOVA) at significance level of $p < 0.05$ with SPSS 19.0 (IBM, USA). Duncan's test was used to detect significant differences. For all the calculations in the present study, measured concentrations were used unless otherwise stated.

4.3 Results

Soil properties

The selected physicochemical properties of the two unspiked soils are shown in Table 4.1. Total concentrations of Cu, Cd, Ni, and Zn were slightly higher in soil II than in soil I. The two test soils varied in pH, texture, OM, and CEC. The pH_{CaCl2} in soil I and soil II was 6.2 and 5.8, respectively. No significant changes in soil pH were observed after metal spiking and soil incubation for two months (Data not shown). Soil I, consisting of 70.5% sand, was classified as sandy loam. Soil II with 53.2% silt as silt loam.

Table 4.1 Selected physicochemical properties of the unspiked soils used for assessing earthworm-ecotype differences in metal accumulation and sensitivity.

Properties	Soil I	Soil II
pH[a]	6.2 ± 0.3	5.8 ± 0.2
Total Cu (mg/kg)	10.6 ± 0.4	26.2 ± 1.7
Total Cd (mg/kg)	0.17 ± 0.05	0.36 ± 0.04
Total Ni (mg/kg)	7.6 ± 0.7	10.9 ± 1.0
Total Zn (mg/kg)	91.0 ± 4.8	132 ± 10.4
Texture[b]	sandy loam	silt loam
Clay (%)	7.0	16.5
Silt (%)	22.5	53.2
Sand (%)	70.5	30.3
OM[c] (%)	5.7 ± 0.2	21.7 ± 1.3
CEC[d] (cmol/kg)	10.1 ± 0.3	38.8 ± 2.2
MWHC[e] (%)	53.5 ± 4.6	69.3 ± 5.7

[a]pH in 0.01 M $CaCl_2$ extract. [b]Determined by the hydrometer method (Pansu and Gautheyrou, 2006). [c]Organic matter content determined by the loss-on-ignition method (Pansu and Gautheyrou, 2006). [d]Cation exchange capacity determined by the ammonium acetate method (Pansu and Gautheyrou, 2006). [e]Maximum water-holding capacity determined by the saturation and gravity drainage method (Pansu and Gautheyrou, 2006).

Metal toxicity

Control earthworm survival was good in all tests, with less than 10% mortality after 28 days of exposure. The LC50 values for the effect of Cu, Cd, Ni, and Zn on the survival of the three earthworm species are listed in Table 4.2. *L. rubellus* was the most sensitive species to all metals, followed by *A. longa* and *E. fetida*. Significant differences in LC50 values between species in each soil were found ($p < 0.05$) (Table 4.2). The maximum differences between the highest and lowest LC50s of Cu, Cd, Ni, and Zn for the three species in soil I were approximately a factor of 18, 18, 3, and 4, respectively, and in soil II 13, 11, 3, and 5, respectively. The difference in species sensitivity was larger for Cu and Cd than for Ni and Zn. Although the LC50s of metals for each species varied between soils, the rankings of species sensitivity (*L. rubellus* > *A. longa* > *E. fetida*) were the same in the two soils which had quite different properties (Table 4.1).

Table 4.2 Median lethal concentrations (LC50s) of Cu, Cd, Ni, and Zn, expressed as total soil concentrations (mg/kg dry soil) and internal concentrations (mg/kg dry body weight), for the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, *and Eisenia fetida* when exposed for 28 days to different metals in the two tested soils.

	LC50s for effect on mortality							
	Total soil Cu	Internal Cu	Total soil Cd	Internal Cd	Total soil Ni	Internal Ni	Total soil Zn	Internal Zn
Soil I								
L. rubellus	72 a (64-80) *	35 (29-40)	108 a (75-156)	382 (309-472)	557 a (497-624)	162 (142-185)	1135 a (998-1291)	1056 (960-1161)
A. longa	111 b (80-153)	26 (22-30)	211 b (173-258)	397 (357-441)	1113 b (911-1359)	N.A.	2504 b (2260-2775)	1376 (1253-1511)
E. fetida	1322 c (1033-1692)	79 (77-81)	1932 c (1659-2250)	698 (634-769)	1824 c (1655-2010)	221 (204-239)	4121 c (3746-4535)	N.A.
Soil II								
L. rubellus	284 a (208-386)	40 (20-79)	277 a (170-450)	233 (138-394)	1803 a (1476-2203)	238 (170-334)	1532 a (1052-2231)	917 (695-1211)
A. longa	1942 b (1779-2119)	79 (68-91)	1231 b (954-1588)	558 (485-643)	3514 b (3092-3994)	N.A.	3828 b (3588-4083)	1332 (1287-1377)
E. fetida	3717 c (3678-3756)	230 (201-258)	2947 c (2707-3209)	772 (722-825)	4977 c (4676-5298)	N.A.	7264 c (6677-7902)	N.A.

*LC50 values (with corresponding 95% confidence intervals in parentheses) marked with the different letters for the three earthworm species within the same soil for each metal are significantly different at $p < 0.05$ by Duncan's test. N.A. = Not applicable

Metal accumulation

Metal concentrations in the earthworms prior to the experiment are shown in Figure 4.1. There were no significant differences in initial internal Cu, Cd and Ni concentration between the earthworms of different species ($p > 0.05$). The Ni concentrations in all three earthworm species were below 0.1 mg/kg dry body weight. The Zn concentration in *E. fetida* (101 mg/kg dry body weight) was significantly lower compared to the other two earthworm species ($p < 0.05$), with 196 and 186 µg Zn/g dry body weight for *L. rubellus* and *A. longa*, respectively.

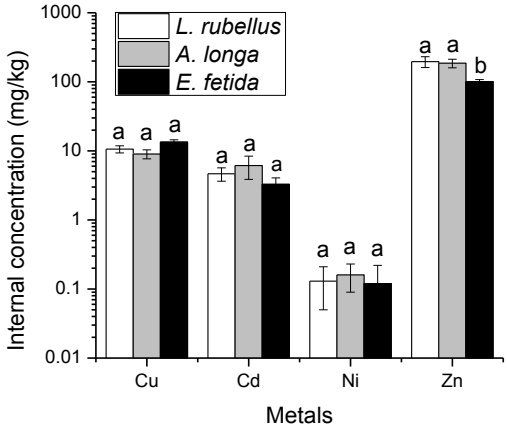

Figure 4.1 Internal metal concentrations in the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, *and Eisenia fetida* before initiation of the accumulation and toxicity testing. Error bars represent the standard errors of the means ($n = 6$). Means marked with the same letter within the same metal are not significantly different at $p < 0.05$ by Duncan's test.

Cu

Accumulation of Cu in earthworms at nontoxic (no mortality is seen) and toxic (mortality is seen) exposure concentrations is shown in Figure 4.2. At low exposure levels (nontoxic concentrations), Cu concentrations in the earthworms increased almost linearly with increasing total soil concentrations. At the same exposure level, internal Cu concentrations followed the order: *L. rubellus* > *E. fetida*> *A. longa*. Cu concentrations in *E. fetida* did clearly level off with increasing exposure concentrations, reaching ca. 80 mg/kg dry body weight in soil I and ca. 240 mg/kg in soil II. For the other two species, no leveling off with concentrations of internal Cu was observed.

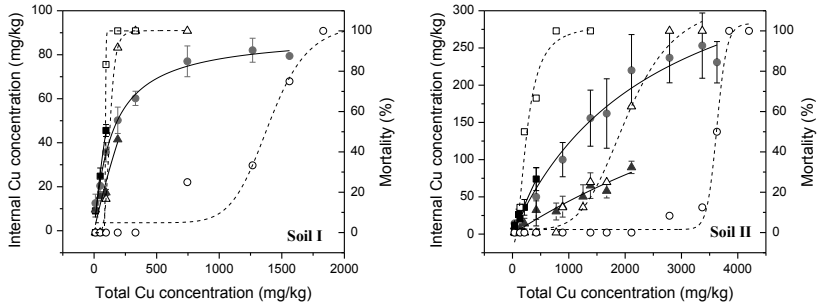

Figure 4.2 Relationships between internal Cu concentration (left Y-axis) in earthworms and earthworm mortality (right Y-axis) on one hand and total Cu concentration in the two soils tested on the other hand, following exposure for 28 days. Solid symbols indicate Cu accumulation in *Lumbricus rubellus* (■), *Aporrectodea longa* (▲), and *Eisenia fetida* (●). Open symbols indicate Cu toxicity in *L. rubellus* (□), *A. longa* (Δ), and *E. fetida* (○). Solid lines are Langmuir model fits (equation 4-1). Dashed lines are logistic toxicity model fits.

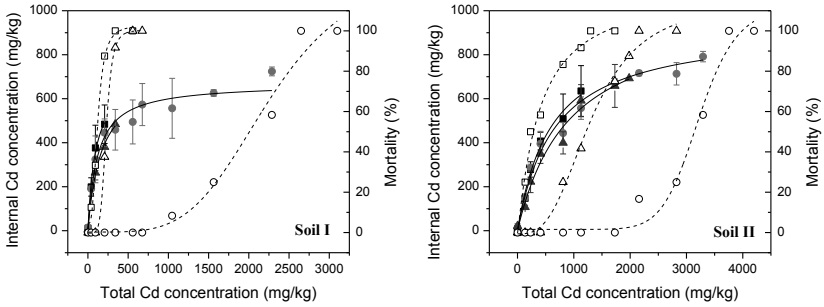

Figure 4.3 Relationships between internal Cd concentration (left Y-axis) in earthworms and earthworm mortality (right Y-axis) on one hand and total Cd concentration in the two soils tested on the other hand, following exposure for 28 days. Solid symbols indicate Cd accumulation in *Lumbricus rubellus* (■), *Aporrectodea longa* (▲), and *Eisenia fetida* (●). Open symbols indicate Cd toxicity in *L. rubellus* (□), *A. longa* (Δ), and *E. fetida* (○). Solid lines are Langmuir model fits (equation 4-1). Dashed lines are logistic toxicity model fits.

The Langmuir model fitted the Cu accumulation data well, with $R^2 > 0.9$ for all species in the two soils (Table 4.3). The maximum body concentration (C_{max}) of Cu for each species differed in each soil. When relating Cu toxicity to internal concentrations, poor model (logistic) fits were obtained with R^2 of 0.49, 0.56 and 0.70 in soil I and 0.62, 0.75, 0.20 in soil II for *L. rubellus*, *A. longa*, and *E. fetida*, respectively. LC50s of Cu based on internal

concentrations are listed in Table 4.2. For each species in the different soils, LC50 values based on internal concentrations were much closer than that based on total soil concentrations.

Table 4.3 Langmuir isotherm parameters relating internal metal concentrations in the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* to total metal concentration in soil, after 28 days exposure.

		Soil I			Soil II		
		L. rubellus	*A. longa*	*E. fetida*	*L. rubellus*	*A. longa*	*E. fetida*
Cu							
	C_{max} [a]	167 (79)[d]	147 (86)	89 (3)	717 (138)	394 (98)	431 (60)
	$\log K_L$ [b]	-2.41	-2.71	-2.17	-3.57	-3.89	-3.49
	R^2_{adj} [c]	0.979	0.906	0.986	0.955	0.936	0.971
Cd							
	C_{max}	712 (71)	705 (53)	661 (31)	946 (97)	1008 (107)	930 (55)
	$\log K_L$	-1.97	-2.21	-2.01	-2.81	-3.07	-2.88
	R^2_{adj}	0.994	0.997	0.924	0.993	0.935	0.952
Ni							
	C_{max}	390559[e](-)[f]	81 (28)	405 (61)	476060[e] (-)	87 (36)	290854[e] (-)
	$\log K_L$	-7.12	-3.03	-3.12	-7.61	-3.49	-6.69
	R^2_{adj}	0.975	0.938	0.919	0.883	0.711	0.766
Zn							
	C_{max}	1961 (348)	2597 (761)	818 (278)	2428 (360)	2704 (707)	530 (151)
	$\log K_L$	-3.00	-3.43	-3.71	-3.55	-3.59	-3.48
	R^2_{adj}	0.937	0.830	0.833	0.915	0.878	0.774

[a] C_{max} = maximum internal concentration (mg/kg dry body weight). [b] K_L = Langmuir constant (kg/mg). [c] Determination coefficients adjusted for the degrees of freedom. [d] Standard error are given in the brackets. [e] Far outside the range of concentrations measured in earthworms and Langmuir isotherm had linear shape. [f] Very wide standard errors.

Cd

Internal Cd concentrations in the three earthworm species generally increased with increasing exposure concentrations in the two soils (Figure 4.3). At any given exposure concentration within the range of 0-500 mg Cd/kg in soil I and 0-1500 mg Cd/kg in soil II, no statistically significant differences in internal Cd concentrations of all three earthworms species were observed ($p > 0.05$) (Figure 4.3). Similar accumulation patterns were observed for each species in different soils. Internal Cd concentrations in *E. fetida* levelled off at approximately 700 mg/kg dry body weight with increasing total Cd concentrations in the two soils. There were no leveling off with concentrations of internal Cd in *L. rubellus* and *A. longa*.

There were no significant differences in C_{max} values of Cd for the three earthworm species in each soil ($p > 0.05$). However, C_{max} values of Cd for each earthworm species differed in the two soils (Table 4.3). LC50 values of Cd expressed as internal concentrations are shown in Table 4.2. Large differences in LC50 values between soils indicated that internal concentration failed to explain the variance in Cd toxicity in the two soils selected.

Ni

At the same exposure level, internal Ni concentrations ranged as follows: *L. rubellus* > *E. fetida*> *A. longa* (Figure 4.4). *A. longa* was found to regulate its internal Ni to a fairly low level (ca. 50 mg/kg dry body weight) at different exposure concentrations. For *L. rubellus* and *E. fetida*, regulation of internal Ni was not observed. For these two species 100% mortality occurred when internal Ni exceeded 250 mg/kg dry body weight in soil I and 300 mg/kg dry body weight in soil II.

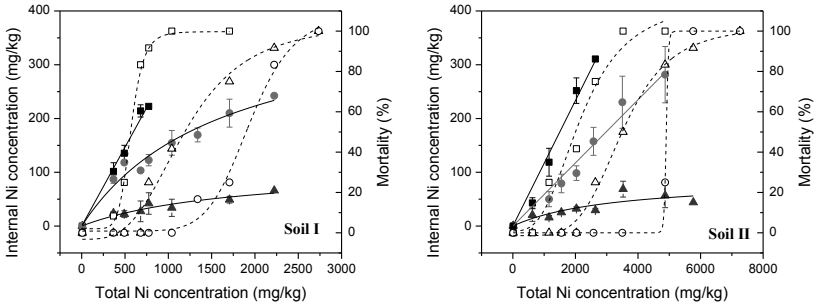

Figure 4.4 Relationships between internal Ni concentration (left Y-axis) in earthworms and earthworm mortality (right Y-axis) on one hand and total Ni concentration in the two soils tested on the other hand, following exposure for 28 days. Solid symbols indicate Ni accumulation in *Lumbricus rubellus* (■), *Aporrectodea longa* (▲), and *Eisenia fetida* (●). Open symbols indicate Ni toxicity in *L. rubellus* (□), *A. longa* (Δ), and *E. fetida* (○). Solid lines are Langmuir model fits (equation 4-1). Dashed lines are logistic toxicity model fits.

The C_{max} values of Ni for *A. longa* were similar in the two soils tested (Table 4.3). Ni concentrations in *L. rubellus* and *E. fetida* increased almost linearly with increasing total Ni concentrations, resulting in a linear Langmuir curve, with unrealistically high values of C_{max}. This shows the Langmuir model was not an optimal model for fitting the accumulation data in these cases. LC50 values of Ni based on internal concentration are listed in Table 4.2. For *A. longa*, the calculation of LC50 based on internal Ni concentrations was not possible because of the relatively constant amount of internal Ni at different exposure concentrations.

Zn

At the same exposure level, internal Zn concentrations followed the order: *L. rubellus* > *A. longa* > *E. fetida* (Figure 4.5). A fairly constant Zn concentration (200-400 mg/kg dry body weight) was found in *E. fetida* in both soils spiked with different levels of Zn, indicating that Zn was highly regulated by *E. fetida*. For the other two earthworms, no obvious regulation of Zn was shown and accumulation patterns of Zn were similar. When internal Zn concentration exceeded 1500 mg/kg dry body weight, both *L. rubellus* and *A. longa* died.

The C_{max} values of Zn and corresponding logK_L values for the three earthworm species are given in Table 4.3. The Langmuir model provided good fits for Zn accumulation in the different earthworm species as reflected by the high R^2 values given in Table 4.3. LC50 values of Zn for *L. rubellus*, expressed as internal concentrations, were similar in the two soils. This was also the case for *A. longa* (Table 4.2). At different exposure concentrations, *E. fetida* maintained Zn at a constant level. It therefore was not possible to calculate LC50 values based on internal concentrations for *E. fetida*.

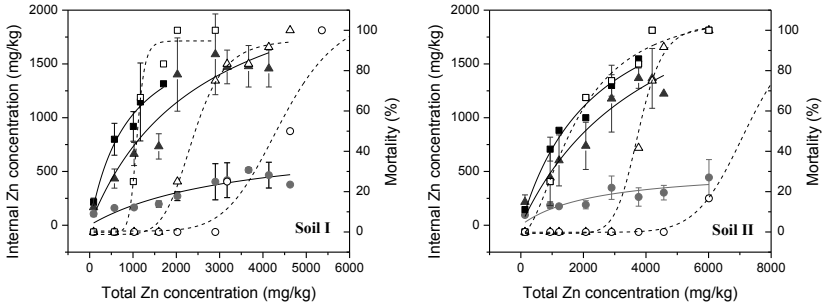

Figure 4.5 Relationships between internal Zn concentration (left Y-axis) in earthworms and earthworm mortality (right Y-axis) on one hand and total Zn concentration in the two soils tested on the other hand, following exposure for 28 days. Solid symbols indicate Zn accumulation in *Lumbricus rubellus* (■), *Aporrectodea longa* (▲), and *Eisenia fetida* (●). Open symbols indicate Zn toxicity in *L. rubellus* (□), *A. longa* (Δ), and *E. fetida* (○). Solid lines are Langmuir model fits (equation 4-1). Dashed lines are logistic toxicity model fits.

4.4 Discussion

Nontoxic and toxic exposure concentrations

Earlier studies on metal accumulation in earthworms mainly focused on relatively low, nontoxic exposure concentrations (Hobbelen et al., 2006; Li et al., 2009; Santorufo et al., 2012). In the present study, Cu, Cd, Ni and Zn uptake by three different earthworm species was investigated at broad concentration ranges, even at levels causing toxicity. From the metal accumulation patterns in each species (Figure 4.2-4.5), it can clearly be seen whether the bioaccumulation was affected by toxic effects or not. Generally, within the range of nontoxic exposure levels, internal metal concentrations of the three earthworm species increased linearly with increasing exposure concentrations. At toxic exposure concentrations, internal metal concentration either leveled off or had a tendency to level off. Therefore, the Langmuir isotherm was chosen to relate internal metal concentrations in the earthworms with (non)toxic exposure concentrations in soil. Contrary to the Freundlich isotherm or linear regression, which just gives a mathematical description of the relation between body concentration and soil concentration, the Langmuir isotherm does have some mechanistic meaning (Borgmann et al., 2004). It provides an indication of the maximum level that may be reached in the earthworms, while it also gives a measure of the uptake affinity (indicated by

K_L) (Van Gestel et al., 2011). The relative high logK_L values of Cd compared to that of Cu, Ni and Zn in the present study indicated a high uptake affinity of the three earthworm species for Cd. The frequent observation of a high concentration plateau for metals in all three species suggested that internal sequestration or detoxification mechanism might be involved (Vijver et al., 2004). In some cases the Langmuir isotherm had an almost linear shape (e.g., *L. rubellus* for Ni). This suggested that earthworms did not possess an active excretion mechanism that may assist in eliminating excess metal from the body (Van Gestel et al., 2011). In this case, metal accumulation reflected the level of external exposure. When relating toxicity to internal concentrations in the surviving earthworms, the obtained LC50 values between soils for each species were much closer to each other than those based on total soil concentrations. This implied that external exposure might not be the main factor but some internal processes (toxicological bioavailability) driving metal toxicity, which is in agreement with previous studies of Peijnenburg et al. (1999) and Vijver et al. (2004). Although internal concentrations did represent the bioavailability much better than total soil concentrations, the internal concentrations cannot explain all toxicity variations between soils especially for those cases where internal metal concentrations of earthworms started to level off when exposure was approaching toxic levels. In those cases, earthworm traits might give more explanatory power.

Traits involved in metal accumulation and toxicity

Traits-based approach has a long history in evolutionary biology, facilitating mechanistic prediction on performance across environmental gradients (Webb et al., 2010). Previously, the results of earthworm ecotoxicological tests were often interpreted from the perspective of bioavailability and toxicity of metals (Hobbelen et al., 2006; Lukkari et al., 2005; Nannoni et al., 2011; Peijnenburg et al., 1999). Only a few studies dealt with the effects of ecophysiological traits of earthworms (e.g., Morgan and Morgan, 1999). However, recently characteristics of organisms received more attention in ecotoxicology, and showed to have explaining power in interpretation of organism vulnerability (sensitivity) to chemicals (Baird et al., 2008).

Following trait-based approaches, organisms are classified in terms of physiological, morphological or life-history traits (Webb et al., 2010). Rubach et al. (2012) have proven experimentally that there are quantitative links between organismal traits and toxicokinetic endpoints following exposure to pesticides. In the present study, the focus was only on the traits that likely might explain the differences as found in the earthworm accumulation and sensitivity to metals (Table 4.4**)**. It should be noted that the traits given in Table 4.4 represent a non-conclusive list and other traits might also be of relevance. For now, there is no clear standard about the selection of traits (De Lange et al., 2013). The earthworms species selected in the present study all belong to the same family (Lumbricidae), but differ in their genus (Pop and Pop, 2004). Within commonly applied ecotoxicity tests with earthworms, *E. fetida* is often used being a representative species for the Lumbricidae group (Spurgeon et al., 2000; OECD, 2004). Measuring the accumulation and toxicity related traits might assist in extrapolating the results over species. Additionally it might give answers on why one species is more sensible to metals than another species, because two species of the same genus may show large differences in sensitivity. Hence it has been argued that taxonomy is not an

inherently informative indicator for prospective effect assessment of metals (Rubach et al., 2010). Uptake and sensitivity related organism characteristics were either measured or collected from literature and linked to the observed accumulation patterns and toxicological responses as seen in our study (see Table 4.4). These selected traits are assumed to make the species react to metal stress differently from each other. Some of the earthworm traits were quantifiable, such as time to maturity, cocoon production, body weight, and length, and others were qualitative, such as calcium glands activity, immune system, food choice and mobility. Traits like cocoon production and respiration rate were included as they are related to the energy flow. Respiration is a measure of the part of the food intake which is converted to heat and hence represents the energy loss from the system (Terhivuo, 1989). More energy spent on reproduction and respiration indicates that less energy can be spent on detoxification strategies (Holmstrup et al., 2011). Food preference behavior was selected as it was suggested that the difference in dietary uptake was an important factor in contributing to the observed differences in metal accumulation between *L. rubellus* and *A. caliginosa*, although other factors are also contributory (Morgan and Morgan, 1999). Since *E. fetida* is artificially cultured for vermicomposting (Domínguez, 2004), it is assumed to be the species preferring the organic-rich food most. Doube et al. (1997) studied the food preference behavior of *A. longa* and *L. rubellus*, and found that after 7 days of exposure, the percentage of food (cow dung) removed by *L. rubellus* was much higher than that by *A. longa* in a cow dung and soil system. Therefore the following order of food choice (organic rich) was estimated: *E. fetida* > *L. rubellus* > *A. longa*. Transporters in membrane (ion channels and transport proteins) of earthworms were estimated to be similar across species based on the findings on aquatic organisms (Niyogi and Wood, 2004). As earthworms have permeable skin and high exposure via the porewater of soils (70-100% for metals) (Vijver et al., 2003), it was assumed that the terrestrial earthworms can be compared to aquatic organisms respecting their uptake. Moreover, general binding ligands and transporters (such as calcium and sodium transporters) in the membrane are inherent to every living cell. Therefore, the extrapolation among different (even aquatic) organisms appears to be valid.

Table 4.4 Differences in the traits probably affecting metal accumulation and toxicity in the earthworm species *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*.

Type of traits	Traits	Quantitive/Qualitative differences between species			Sources
		L. rubellus	*A. longa*	*E. fetida*	
Life-history	time to maturity (months)	6-7	4	2-3	Butt, 1993; Edwards and Bohlen, 1996
	cocoon production (n/year)	106	18.8	> 200	Edwards and Bohlen, 1996; OECD, 2004
Ecological	habitat (depth) (cm)	0-5	> 10	5-10	Bouché, 1977
	organic rich (food choice)	*E. fetida* > *L. rubellus* > *A. longa*			Doube et al., 1997; Domínguez, 2004
	mobility	good	limited	reasonable	Edwards and Bohlen, 1996
Physiological	calcium glands activity	*L. rubellus* and *E. fetida* > *A. longa*			Piearce, 1972; Spurgeon and Hopkin, 1996
	transporters in membrane	*L. rubellus* ≈ *A. longa* ≈ *E. fetida*			Estimated based on the aquatic organisms (Niyogi and Wood, 2004)
	respiration rate	*L. rubellus* and *E. fetida* > *A. longa*			Uvarov and Scheu, 2004
	mucus production	*E. fetida* > *L. rubellus* and *A. longa*			Zhang et al., 2009
	Immune-competent cells	*E. fetida* > *L. rubellus* and *A. longa*			Plytyce et al., 2011a; 2011b
Morphological	length (mm)	60-90	90-140	70-110	present study
	weight (mg)	700-900	800-1000	600-800	present study
	surface area/ mass	*L. rubellus* > *E. fetida* > *A. longa*			Spurgeon and Hopkin, 1996; Spurgeon et al., 2000

Do earthworm traits provide a clue for predicting metal toxicity?

In the present study, species sensitivity to Cd, Cu, Zn and Ni decreased in the following order: *L. rubellus* > *A. longa* > *E. fetida*. Langdon et al. (2005) reported that both *L. rubellus* and *A. caliginosa* were more sensitive to Pb than *E. andrei*. Spurgeon et al. (2000) found that *L. rubellus* and *A. caliginosa* were more sensitive to Zn than *L. terrestris* and *E. fetida.* Based on the traits theory, differences in sensitivity between species might be explained as follows. The epigeic earthworm *E. fetida* (or *E. andrei*) feeds almost entirely on the soil surface on organic matter (cow manure in the present study), whereas the endogeic *A. caliginosa* and anecic *A. longa* would be more exposed to metals via the soil porewater as they live and feed in the soil (Langdon et al., 2005). Some authors suggested that the differences in sensitivity are partially attributed to the activity of the calciferous glands in earthworms as calcium is involved in the sequestration and elimination of many metals through the chlorogogenous tissue (Piearce, 1972; Morgan and Morgan, 1988; Spurgeon and Hopkin, 1996; Spurgeon et al., 2000). *E. fetida*, which has active calciferous glands, was least sensitive, while *A. caliginosa and A. longa* having less active or inactive calciferous glands (Piearce, 1972; Spurgeon et al., 2000), were more sensitive. *L. rubellus* was shown to be more sensitive than the other species despite that it has active calciferous glands. The relatively small size of *L. rubellus* with a large ratio of surface area to mass (potential high uptake rate) probably serves as an explanation, as some studies have shown that the rate of uptake, not the concentration itself, was the superior predictor of metal toxicity (e.g., Van Straalen et al., 2005). Besides, the least sensitive earthworm species *E. fetida* has higher immune ability and mucus production compared to the other species. Plytycz et al. (2011a; 2011b) reported that earthworms have immune systems displaying striking inter-species differences. For instance, eleocytes (one type of immune-competent cells) are very seldom present in species belong to *Lumbricus* and *Aporrectodea* genera, but are common in species such as *E. fetida*. Overall, ranking of species sensitivity cannot be quantitively linked to one specific trait. It is most likely that each species possesses different trait combinations to cope with metal stress (De Lange et al., 2013).

Do earthworm traits provide a clue for predicting metal accumulation?

The amount of metal accumulated in earthworms at the same exposure concentration followed the order: *L. rubellus* > *E. fetida* > *A. longa* for Cu and Ni, *L. rubellus* ≈ *E. fetida* ≈ *A. longa* for Cd, and *L. rubellus* > *A. longa* > *E. fetida* for Zn. Assuming the traits theory applies in our cases, a set of traits can be related to the metal accumulation ranking in the earthworm species tested (Table 4.4). In addition to good mobility (Edwards and Bohlen, 1996), *L. rubellus* has larger surface area/mass ratio (thus higher potential uptake rate as suggested by Spurgeon et al. (2000)) than the other two species. This may cause *L. rubellus* to be more exposed to metals, resulting in higher internal metal concentrations compared to the other species. The relatively low accumulation of metals in *A. longa* might be explained by its limited mobility (which was also observed in the present study) and thus less exposure as this species was inclined to stay in their deep burrows (Emmerling and Strunk, 2012). As the traits listed in Table 4.4 differed largely between the different species, it was surprising that the three earthworm species accumulated similar amounts of Cd at the same exposure concentrations. This might be attributed to the high uptake affinity of earthworms for Cd (as

reflected by logK_L). Traits that showed to be similar over all species were transporters in membrane (i.e., ion channels and transport proteins), which determines uptake affinity for different metals (Niyogi and Wood, 2004). Implementing the traits theory for quantitatively explaining all the observed accumulation patterns of different earthworm species was shown to be difficult.

Earthworms did not appear to accumulate Cu at high concentrations even when exposed to heavily contaminated soils. This is in agreement with the findings by Nannoni et al. (2011), and is probably caused by the relatively high intrinsic earthworm toxicity of Cu. Although Cu concentrations in the three earthworm species were almost the same before initiation of the accumulation and toxicity testing, internal Cu concentrations after 28 days were always higher in *L. rubellus* than in *E. fetida* and *A. longa* at the same exposure levels. Internal Cd concentrations were identical for the three species at approximately 500 mg Cd /kg in soil I and 1000 mg Cd/kg in soil II. At higher soil concentrations, the internal Cd concentrations in *E. fetida* strongly leveled off while 100% mortality of *L. rubellus* and *A. longa* occurred. This suggests that *E. fetida* is less sensitive to Cd than *L. rubellus* and *A. longa* not because it can tolerate more Cd inside the body but because it is able to more effectively prevent uptake of Cd from soil. Langdon et al. (2005) reported no significant differences in internal Pb levels in *L. rubellus*, *A. caliginosa* and *E. andrei* exposed to same soil Pb concentrations. They claimed that the relatively small size of the soil container likely prevented the traits of different earthworms manifesting itself. Nannoni et al. (2011) found similar internal concentrations of Cu, Cd, Zn, Pb, As, and Sb in the endogeic earthworms *A. rosea* and *A. caliginosa* collected from 15 contaminated sites. They suggested that earthworm species belonging to the same ecophysiological group have a similar tendency to accumulate metals. However, it has been argued that two species that are strongly taxonomically related do not necessarily respond similar to metals and that the taxonomic distance can only give a hint (Rubach et al., 2010). Internal Ni concentration was regulated at a fairly low level by *A. longa*, which was different from *L. rubellus* and *E. fetida* who did not show this pattern, as reflected by the linear Langmuir curves. This pattern was Ni-specific. There are comparatively few data on Ni accumulation in *A. longa*. Yan et al. (2011) reported that internal Ni concentrations ranged from 109 to 286 mg/kg dry body weight in *E. fetida* exposed for 28 days to the highest Ni concentrations (1000 mg/kg) spiked into a range of Chinese soils. Ni concentrations in *E. fetida* in the present study were at the same level. Generally, the maximum level of Ni that may be reached in all the three earthworm species was lower than that of Cd and Zn. This could be due to the mode of action of Ni. This metal is known to cause skin irritation, which may lead to damage to the epithelium of both the gut and the body wall (Muñoz and Costa, 2012). As a consequence, uptake of the metal may be hampered by its damaging effect to the membranes that are responsible for its uptake. Internal Zn levels in *E. fetida* were maintained at a constant level (ca. 250 ug/g dry body weight) at different exposure concentrations. This finding is in agreement with earlier observations showing that the body concentration of Zn in *E. fetida* is regulated at a fairly constant level, regardless of the Zn concentration of the soil (Lock and Janssen, 2001; Lukkari et al., 2005). This may be explained by the rapid Zn elimination in *E. fetida* (Spurgeon and Hopkin, 1999). *L. rubellus* and *A. longa* showed to have accumulation patterns that could be described by the Langmuir isotherm. When the internal Zn

concentration of these two species exceeded 1500 mg/kg dry body weight, 100% mortality occurred regardless of the soil in which the earthworms were exposed. Previous studies reported that Zn concentrations in *L. rubellus* ranged from 394 to 3873 mg/kg dry body weight (Morgan and Morgan, 1988). At such high internal exposure levels, earthworms must either store Zn in nontoxic forms (e.g., binding to specific metal-binding proteins, and storage in granules) (Vijver et al., 2004) or suffer from Zn toxicity (Hobbelen et al., 2006).

Earthworms have different capacities to accumulate metals. Generally, the accumulation patterns of the different metals were not straightforwardly related to the different earthworm species or one specific trait. Empirical traits-based approaches face the challenge of how to make a priori selection of traits to be included for analysis and quantitatively integrating these traits.

4.5 Conclusions

From this study, it can be concluded that metal accumulation patterns and sensitivity differed between earthworm species. Variations in metal accumulation and sensitivity of species might result from differences in ecological strategies and from physiological differences in detoxification and elimination strategies. At the same exposure concentration, internal Cu and Ni concentrations were highest in *L. rubellus*, intermediate for *E. fetida*, and lowest in *A. longa*. For internal Zn concentrations, the order was *L. rubellus* > *A. longa* > *E. fetida*. There were no significant differences in internal Cd concentrations between species. Species sensitivity to all metals followed the same order: *L. rubellus* > *A. longa* > *E. fetida*. In the context of risk assessment, the results obtained with *E. fetida* should be interpreted with caution taking into account the low sensitivity of this species. Although traits-based approach showed to be powerful in explaining evolutionary processes, and is promising in ecotoxicology, quantitative interpretation of our results with traits was difficult. Based on our findings, likely more than one trait is involved in the processes of metal accumulation and toxicity in earthworms. More research is needed on these aspects (e.g., identifying new traits), enabling ecologically-sound extrapolation of ecotoxicological data across species of earthworms.

Chapter V

Interactions Of Cadmium And Zinc Impact Their Toxicity To The Earthworm *Aporrectodea Caliginosa*

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Abstract

Individual and binary mixture effects of Cd and Zn on mortality of the earthworm *Aporrectodea caliginosa* were investigated in one soil type. Mutual, systemic interactions of Cd and Zn were assessed over a broad range of concentration combinations. Exposures were expressed as measured total concentrations in soil, porewater concentrations, and $CaCl_2$-extractable concentrations. The Cd-Zn and Zn-Cd interactions in soil were estimated by comparing partition coefficients (K_d) of one metal in the presence and absence of the second metal. Simple patterns of mixture toxicity (additive, antagonistic or synergistic) were evaluated by the toxic unit approach. The more complex patterns (dose ratio-dependent or dose level dependent) were quantified by the MIXTOX model. The partitioning of Cd and Zn between soil and porewater was affected neither by their concentration nor presence of the other metal, and metal partitioning remained linear at all exposure levels tested. The effect of the Cd and Zn mixtures on mortality of *A. caliginosa* was mainly antagonistic, and the magnitude of antagonism (1.1 to 2.7 toxic units) was dependent upon both the relative concentrations of Cd and Zn and the concentration magnitudes. Interactions of both metals occurred at the organism level and were manifested in terms of effects (28-day median lethal concentrations). This study highlights the importance of identifying the relative influence of various interactions from external exposure to internal assimilation in evaluating mixture toxicity.

5.1 Introduction

Cadmium (Cd) is highly toxic and has no known physiological function. Zinc (Zn) is an essential micronutrient for most organisms, but causes toxicity at high concentrations (Rüdiger and Ralf-Rainer, 2010). Cadmium and zinc have many similar physicochemical properties as Cd appears below Zn in group IIB of the periodic table. They are usually found together in nature and compete with each other for various ligands owing to structural electronic similarities (Das et al., 1997). The fact that Cd is non-essential metal and Zn essential makes this association interesting as it raises the possibility that the toxicity of one metal may be prevented or aggravated in the presence of the other. Therefore, an understanding of the mutual interactions of Cd and Zn in mixtures and the impact on organisms is important.

The toxicity of individual metals to a wide range of soil dwelling organisms has been extensively investigated and is well documented (Das et al., 1997; Fitzpatrick et al., 1996; Spurgeon et al., 2000). However, as contamination in the environment generally consists of mixtures of varying composition, investigations on singular metal effects may have little real environmental relevance. Multiple metals can interact with each other in varying degrees to produce joint toxicity, which may be similar to, stronger or weaker than expected from exposure to a single metal (Zidar et al., 2009). In addition, interactions between different components may also depend on the mixture concentration or ratios of concentrations (Jonker et al., 2005). It is unrealistic and infeasible to assess every possible mixture combination. Toxicologists have therefore developed two prominent models for predicting mixture toxicity: the concentration addition and independent action models (Altenburger et al., 2004). Concentration addition model is based on the assumption that mixture constituents with a similar mode of action will act additively, while independent action model assumes that mixture components have dissimilar action modes. Mixture effects can be quantified using these two models (Jonker et al., 2005).

In the soil ecosystem, mixture toxicity is complex to study because interactions of metals can occur at various levels (Dickson et al., 1994), e.g. the exposure level, the uptake level, the target level (Rüdiger and Ralf-Rainer, 2010) and the internal pathway of detoxification (protective effect) (Vijver et al., 2011). For the purpose of toxicity assessment of metal mixtures, it is crucial to acknowledge all relevant interaction levels.

Specific toxic effects of Cd have been related mainly to Cd-induced oxidative damage and the interference with essential elements including Zn (Brzóska and Moniuszko-Jakoniuk, 2001; Jihen et al., 2010). Cadmium may displace Zn in a number of biological processes due to a greater affinity for S-ligands and N-donors. Zinc plays a fundamental role in various metabolic processes (Brzóska and Moniuszko-Jakoniuk, 2001). A deficiency in zinc can cause malfunctions of some organs and functions of organisms. However, excess Zn may also induce toxic effects (Rüdiger and Ralf-Rainer, 2010). It is generally agreed upon that Zn has beneficial actions against either Cd-induced oxidative stress (Jihen et al., 2009) or other Cd-induced toxic effects (Jihen et al., 2008). Increasing Zn supply may reduce Cd sorption and accumulation (Brzóska and Moniuszko-Jakoniuk, 2001), whereas Zn deficiency can intensify Cd accumulation and toxicity (Tang et al., 1998).

Mutual interactions of Cd and Zn do not only occur at the organism level. In soil, metals compete for sorption sites, resulting in competition at the exposure level and a specific distribution of interacting metals over different metal species. Previous studies have suggested that for soil organisms exposure takes place via the porewater or that uptake of metal is mediated by a porewater related route (Janssen et al., 1997; Vijver et al., 2003). The porewater hypothesis proposes that free metal ions in porewater are the major toxic species available for uptake by soil organisms (Plette et al., 1999; Van Gestel and Koolhaas, 2004).

Due to the interactions at the exposure level, bioavailability cannot be ignored in assessing mixture toxicity. It is widely recognized that total concentration is not sufficient to properly quantify metal bioavailability in soil (Sauvé et al., 2000). The bioavailable fraction is the amount of a metal in soil that is either available or potentially available for uptake by organisms (Peijnenburg et al., 2007). By using the concentration of bioavailable metal fraction for exposure assessment for soil organisms, more insight into mixture effects could be obtained. Alternative methods to establish the bioavailable metal content of a soil, like porewater collection or extraction with 0.01 M $CaCl_2$, seem to provide a feasible addition in this respect (Peijnenburg et al., 2007; Peijnenburg and Jager, 2003). Porewater metal concentrations are easily biological available within a short time span (Peijnenburg and Jager, 2003; Vijver et al., 2003). The fraction associated with $CaCl_2$ extraction is often supposed to reflect the potentially bioavailable metal concentrations, including soluble metal pools and labile metal bound to solid phases in soil matrix (Houba et al., 1996; Kalis et al., 2008). To get the full picture of the bioavailable metal fraction, a number of abiotic modifying factors (e.g. pH, competing ions, and complexing ligands) in soil should be considered as well (Peijnenburg and Jager, 2003).

This study aims to quantify the impact of Cd addition on chronic Zn toxicity with LC50 (concentration producing 50% mortality) as the endpoint for the earthworm *A. caliginosa* and vice versa, and to determine where the interactions possibly occur and their influence on the toxicity pattern of binary metal mixtures.

Soil pH is generally accepted as the dominant factor influencing metal bioavailability and toxicity (Sauvé et al., 2000). In this experiment, where pH was relatively constant, competition from Cd and Zn is presumably the main variable with respect to metal bioavailability and toxicity. It is hypothesized that competition and replacement between metals will take place at both the exposure and the organism level (sites of toxic action) (Brzóska and Moniuszko-Jakoniuk, 2001). Toxicity of Zn increases with increasing Cd concentration, because the fraction of biological action sites bound to Cd will increase. In the case of fixed Cd concentrations, a protective effect of Zn on Cd toxicity will be expected under Zn addition, as many studies have reported beneficial actions of Zn against Cd induced oxidative stress or other toxic effects (Jihen et al., 2009; 2010).

5.2 Materials and methods

Soil

Soil was sampled from a nonpolluted grassland in Boxtel, The Netherlands. The physicochemical properties of the soil sample tested are shown in Table S5.1 (Supporting Information). The soil was air dried, homogenized and passed through a 2 mm sieve before

use. It was then spiked with a wide range of concentrations of Cd alone and Zn alone, and different combinations of a mixture of Cd and Zn. The metals were added as their acetate-salts (Acros Chemicals, Leicestershire, UK; purity 96%) and allowed to equilibrate for at least two months at 35 ˚C to eliminate the acetate by mineralization. A pilot study showed that the net result of acetate mineralization and hydrolysis of the Cd and Zn ions released induces only marginal effects on soil pH.

Toxicity tests

The earthworm *Aporrectodea caliginosa* was used in the toxicity bioassays. This endogeic earthworm lives in the upper 25 cm of the soil and feeds on decayed organic matter and bits of mineral soil (Spurgeon et al., 2000). It spreads over large areas in the Netherlands and other European countries (Spurgeon et al., 2000; Vijver et al., 2003). Amongst others, it has the ability to survive in water-saturated soils and can be used in water-only exposures. As such it is an earthworm species that is often used in laboratory testing (Khalil et al., 1996; Spurgeon et al., 2000; Vijver et al., 2003;). Mature earthworms were collected from a sandy, uncontaminated soil located in Liempde, The Netherlands. The earthworms, each weighing from 1200 to 2000 mg (5 to 8 cm in length), were kept in the laboratory for at least one week in the soil from which they originally were collected at 15 ± 2 ℃. This pre-incubation period was necessary to allow the earthworms to get adapted to the exposure conditions. Thereafter they were exposed to Cd alone, Zn alone, or a mixture of Cd and Zn for 28 days. Cadmium toxicity was tested at twelve concentrations ranging from nominal 0 to 1000 mg Cd/kg soil. Fifteen concentrations of Zn were set ranging from nominal 0 to 1500 mg Zn/kg soil. All concentrations are given as dry weights. Cadmium and zinc mixtures were designed as follows: at each constant Cd concentration, different concentrations of Zn were added, or different concentrations of Cd were added at each fixed Zn concentration. Detailed concentration combinations of the spiked Cd and Zn are illustrated in Figure 5.1. Exposures were conducted in plastic boxes filled with 350 g of the different treatments of soils. Four earthworms were used in each treatment. A limited number of replicates were conducted as LC50 values of duplicates were within 8% deviation. All soils were kept at 80 % maximum water holding capacity during the experiment. Continuous illumination (1500 Lux) was used to avoid a possible escape of earthworms from the containers. Earthworms were not fed during the experiment. Mortality was checked after 28 d of exposure. Earthworms were counted as dead if they did not respond to a gentle mechanical stimulus. The number of earthworms that had survived and their body weights were recorded. In the control (no metal added), mortality of the earthworms was zero and no significant loss of weight ($p > 0.05$) was observed after 28 d of exposure.

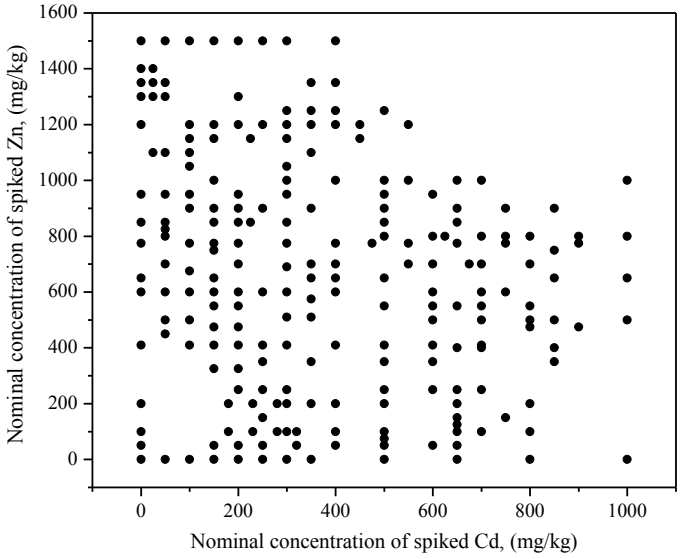

Figure 5.1 Concentration combinations of Cd and Zn spiked for mixture exposures

Chemical analysis

After 28 d of exposure, soil samples were dried at 40℃ and sieved (< 2 mm). Total metal concentration of the soil was measured after digestion with *aqua regia* in a microwave oven (Milestone ETHOS900, Italy). For quality assurance purposes, the certified reference material CRM 483 (the Community Bureau of Reference, BCR, Brussels, Belgium) was used for each of 30 samples. Recovery was 95% ± 14%. Organic matter content was determined by heating soil that had been dried for 3 h at 100 ℃ to 550 ℃ for 3 h. Clay content and cation exchange capacity (CEC) were determined by the hydrometer method and the ammonium acetate method respectively (Pansu and Gautheyrou, 2006). After toxicity tests, porewater was sampled by means of centrifugation and subsequent filtration through 0.45 μm acetate filter of soil samples at 80% maximum water holding capacity (15 ℃). Some soil samples were taken before, during and after the tests. No noteworthy differences in metal contents of porewater were observed among different sampling periods. Soil pH was measured in the porewater samples using a Metrohm 691 pH Meter equipped with a combination pH glass electrode (Metrohm AG, Herisau, Switzerland). Dissolved organic carbon (DOC) in the porewater was measured with a TOC/DOC analyzer (TOC-VCSH, Shimadzu, Kyoto, Japan). Chemical extraction was performed using 10 g of soil in 10 ml of 0.01 M $CaCl_2$ and the suspension was shaken for 24 h. Metal concentrations were determined by inductively coupled plasma atomic emission spectroscopy (Spectro Analytical Instruments, Kleve, Germany). The detection limits for Zn and Cd were 0.2 μg/L and 0.1 μg/L, respectively.

Data analysis

Median lethal concentrations (LC50) were calculated by probit analysis using software SPSS 16.0 (IBM, Chicago, USA). In case no exact LC50 could be calculated because of a very steep dose-response relationship, a binominal approach was used in which the LC50 was set equal to the arithmetic average of the maximum concentration causing 0% mortality and the minimum concentration causing 100% mortality. Relationships between Cd and Zn concentrations in total soil, porewater and in $CaCl_2$-extracts were determined by means of general linear regression using software GraphPad Prism 5.0 (GraphPad Software Inc., California, USA).

To address the toxic effects in the mixtures, the observed effect was compared to the expected effect of mixtures that calculated from the single metal exposure toxicities. This procedure was based on the concentration addition model:

$$TU_{mix} = \sum_{i=0}^{n} \frac{c_i}{EC_{xi}} \tag{5-1}$$

where c_i (mg/kg) is the concentration used for metal i in the mixture and EC_{xi} (mg/kg) is the standard effect concentration of metal i. TU_{mix} is therefore a dimensionless ratio that can be regarded as the sum of each toxic unit (TU). In the present study, mortality was selected as the endpoint in the binary mixtures of Cd and Zn, so the above equation can be expressed as:

$$TU_{mix} = \frac{c_{Cd}}{LC50\,of\,single\,Cd} + \frac{c_{Zn}}{LC50\,of\,single\,Zn} \tag{5-2}$$

where c_{Cd} and c_{Zn} are the experimental concentrations at which we observed 50% mortality. In a mixture of Cd and Zn, strict additivity occurs when the TU_{mix}=1. Values of TU_{mix} exceeding 1 describe antagonism, while values below 1 denote synergy. When manually calculating TU_{mix}, additivity was allowed to deviate up to 10% before the effect of a mixture was assigned as being either antagonistic or synergistic. This deviation was the conservative choice considering the actual experimental condition.

More complex calculations using the concentration addition model including dose ratio (DR) and dose level (DL) were analyzed using the MIXTOX modules (Jonker et al., 2005). Hence, deviation from additivity could then be quantified using significance tests. The binary TU_{mix} model was generalized to

$$\frac{c_1}{f_1^{-1}(Y)} + \frac{c_2}{f_2^{-1}(Y)} = \exp(G) \tag{5-3}$$

Where c_1 and c_2 indicate the concentration of individual metals in the mixture, $f_1^{-1}$and f_2^{-1} indicate the inverse dose-response functions of the single metal in the mixture. Y defines the biological response and G was an extent function used to quantify deviation from additivity. Extra parameters a and b were introduced into the model using a stepwise approach to describe deviation. The model was fitted to the data using the method of maximum likelihood while minimizing the sum of the square residuals. The statistical significance of the improvement in fit from the extended parameters was obtained through Chi-square (χ^2) tests. The biological interpretations of the extra parameters are listed in Table 5.1 and can also be found in Jonker et al. (Jonker et al., 2004; 2005).

Table 5.1 Interpretation of parameters (*a* and *b*) that define the interaction patterns in MIXTOX model (adapted from Jonker et al. 2005)

Pattern	Parameter	Value	Interpretation
Synergy or antagonism(S/A)	*a*	<0	Synergy
		>0	Antagonism
Dose-ratio dependence(DR)	*a*	<0	Synergy, except for those mixture ratios where significant positive b_i indicate antagonism
		>0	Antagonism, except for those mixture ratios where significant negative b_i indicate synergy
	b_i	>0	Antagonism where the toxicity of the mixture is caused mainly by toxicant *i*
		<0	Synergy where the toxicity of the mixture is caused mainly by toxicant *i*
Dose-level dependence(DL)	*a*	<0	Synergy at low dose level and antagonism at high
		>0	Antagonism at low dose level and Synergy at low
	b_{DL}	>1	Change at lower dose level than the EC50
		=1	Change at EC50 level
		0~1	Change at higher dose level than the EC50
		<0	No change, but the magnitude of synergy or antagonism is dose level dependent

5.3 Results

Soil and porewater properties

The organic carbon content of the soil used was 5.7 ± 0.2 %. All values are presented as arithmetic means of three replicates and standard deviation unless stated otherwise. Clay content accounted for 7.0 ± 0.6 % and CEC was 10.1 ± 0.3 cmol/kg. The pH of the porewater from the original soil sample was 6.67 ± 0.15. The addition of Cd, or Zn, or a mixture of Cd and Zn had negligible effect (usually < 0.3 unit) on the porewater pH (Data not shown). In all metal-spiked soil samples, the porewater pH was 6.49 ± 0.28, which was not statistically different ($p > 0.05$) from the pH prior to spiking. The DOC concentration in the porewater of the untreated soil was 171.4 ± 9.8 mg/L. Under the influence of metal addition, the concentration of DOC decreased to an average of 122 ± 13.1 mg/L after 28 d of exposure.

For both metals, the nominal total concentrations correlated linearly with the concentrations measured in total soil, porewater and $CaCl_2$-extracts (see Table 5.2). In this experiment, not all metal concentrations were measured. Instead, the missing values were estimated based on the nominal total concentration using the equations listed in Table 5.2. Measured total metal concentrations in soil were in agreement with the nominal total concentrations (usually <10 % deviation). The measured total Cd concentration showed a good linear relationship with the porewater Cd concentration and the amount of $CaCl_2$-extractable Cd. The measured total Zn concentration also was significantly correlated with the Zn concentration in the porewater ($p < 0.0001$) and the Zn concentration in $CaCl_2$ extracts

($p < 0.0001$). Addition of pH or DOC as explanatory variables did not significantly improve the regression equation presented in Table 5.2 (Data not shown).

Table 5.2 Linear regression relationships between nominal total concentrations (mg/kg), measured total concentrations (mg/kg), porewater concentrations (μg/L) and $CaCl_2$-extractable concentrations (mg/kg) of metals

Equations	R^{2*}	n	p	F
log(measured total Cd)=0.93 log(nominal Cd)+0.21	0.95	115	<0.0001	2300
log(porewater Cd)=1.04 log(nominal Cd)+0.10	0.81	95	<0.0001	395
log($CaCl_2$-extr. Cd)=1.03 log(nominal Cd)-0.57	0.82	166	<0.0001	763
log(porewater Cd)=1.17 log(measured total Cd)+0.65	0.84	91	<0.0001	453
log($CaCl_2$-extr. Cd)=1.04 log(measured total Cd)−0.66	0.67	95	<0.0001	185
log(measured total Zn)=0.70 log(nominal Zn)+0.99	0.92	116	<0.0001	1372
log(porewater Zn)=0.82 log(nominal Zn)+1.58	0.81	95	<0.0001	397
log($CaCl_2$-extr. Zn)=0.79 log(nominal Zn)−0.08	0.77	171	<0.0001	567
log(porewater Zn)=1.19 log(measured total Zn)+0.37	0.83	91	<0.0001	440
log($CaCl_2$-extr. Zn)=1.08 log(measured total Zn)−1.03	0.74	96	<0.0001	262

*R^2 is the coefficient of determination; n indicates the number of data points; p indicates the statistical significance level; F is the value of F test.

Partitioning of Cd and Zn mixtures

The mutual interactions of Cd and Zn at the exposure level were determined in a direct way, by comparison of partition coefficients of one metal in the presence and absence of the second metal. The partition coefficient is usually used to evaluate the partitioning of metal between the soil solid phase and solution phase. It can be defined as the ratio of the measured total concentration to the porewater concentration (K_d) (Sauvé et al., 2000), the ratio of the measured total concentration to the $CaCl_2$-extractable concentration (K_d') (Luo et al., 2006), or the ratio of the $CaCl_2$-extractable concentration to the porewater concentration (K_d'') (Gooddy et al., 1995). In the present study, all the above three forms of partition coefficients were adopted in order to provide more reliable evaluation of metal partitioning.

Partition coefficients of Cd and Zn presented singly and in mixtures are shown in Figure 5.2. In single metal exposures, the effects of metal concentrations on metal partitioning were negligible as the slopes were close to 0 (linear regression equations: K_d (Cd) = − 0.009 total Cd + 83.0 ($p > 0.05$, $n = 11$), K_d (Zn) = − 0.009 total Zn + 115.1 ($p > 0.05$, $n = 9$)). In the mixtures, partitioning of Zn was not significantly different from that in the treatments of Zn alone ($p > 0.05$). Partitioning of Cd was similar. The K_d values for Cd and Zn were on average 91±36 L/mg (n=91) and 141±57 L/mg (n=91) respectively. Nearly all values of K_d were within 50 to 125 L/kg for Cd and 50 to 200 L/kg for Zn (Figure 5.2, A, B). Values of K_d' (Figure 5.2, C, D) and K_d'' (Figure 5.2, E, F) for both metals mostly fell within a relatively narrow range. For all three forms of partition coefficients, the highest values were around 3 to 4 times higher than that of the lowest. Data points were evenly distributed between the highest and the lowest values. Metal partitioning into porewater, into $CaCl_2$ extracts and between porewater and $CaCl_2$ extracts was affected neither by their concentration nor presence of the other metal.

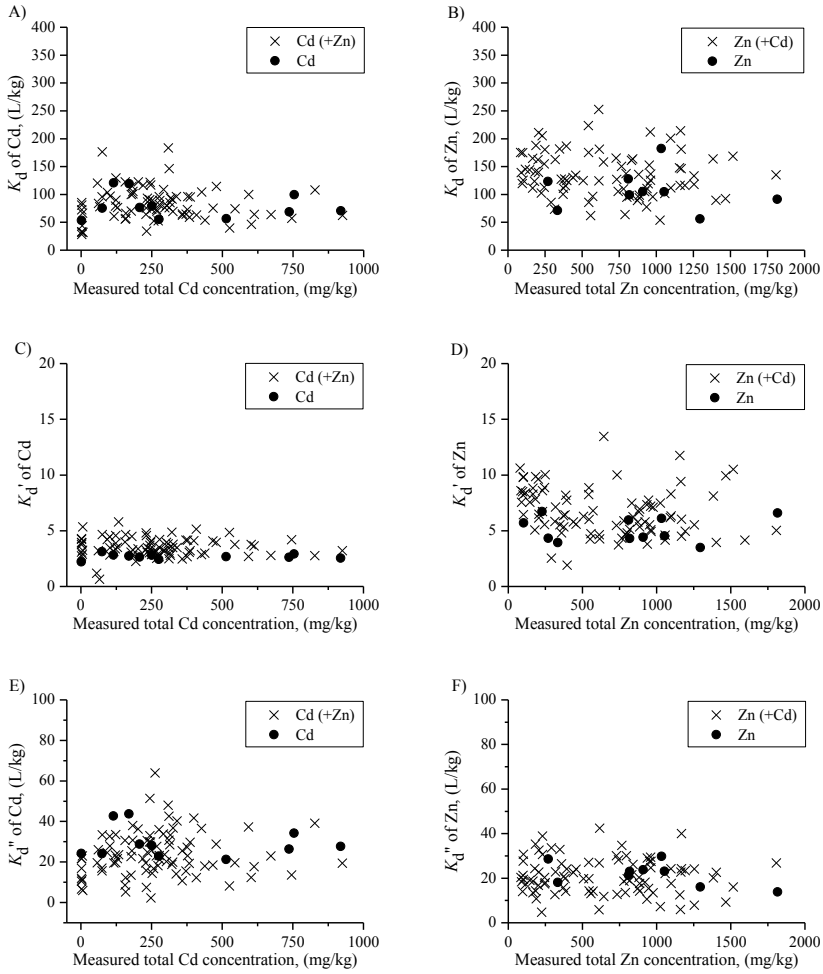

Figure 5.2 Partition coefficients of Cd and Zn presented singly (•) or in mixtures (×). "Cd (+Zn)" represents Cd partitioning in the presence of Zn of the whole concentration range studied, while "Zn (+Cd)" represents Zn partitioning in the presence of Cd of the whole concentration range studied. Partition coefficients were calculated as follows: the ratio of the measured total concentration to the porewater concentration (K_d, L/kg) (A, B), the ratio of the measured total concentration to the $CaCl_2$-extractable concentration (K_d', dimensionless) (C, D), and the ratio of the $CaCl_2$-extractable concentration to the porewater concentration (K_d'', L/kg) (E, F).

Single metal toxicity

Earthworm mortality increased with increasing total concentrations of each metal tested (Table S5.2, Supporting Information). Similar trends were also found when porewater concentration or $CaCl_2$-extractable concentration was assumed to be the exposure pathway.

Values of LC50 of Cd alone and Zn alone for *A. caliginosa* on the basis of measured total, porewater and $CaCl_2$-extractable concentration are shown in Table 5.3. Cadmium caused higher mortality than Zn in *A. caliginosa* when present at the same concentration. Here, the relative toxicity of Cd was approximately 7 times higher than Zn on a molar basis (by dividing measured total concentration to molecular weight).

Table 5.3 Median lethal concentrations (LC50) of Cd, Zn for *Aporrectodea caliginosa* in single metal toxicity tests.

Expressions of exposure	LC50 (95% confidence interval)	
	Cd	Zn
Measured total (mg/kg)	344 (317-375)	1499 (1318-1592)
Porewater (μg/L)	4890 (4155-5416)	13588 (12850-14001)
$CaCl_2$-extractable (mg/kg)	112 (93-137)	317 (279-353)

Impact of Cd on Zn toxicity

Changes in 28 d LC50 values of Zn to *A. caliginosa* at various Cd levels are presented in Figure 5.3. Values of LC50 of Zn, expressed on the basis of measured total, porewater and $CaCl_2$-extractable concentrations, were plotted against added Cd concentration of the three corresponding Cd fractions. Generally, an elevation in Cd content coincided with a reduction in LC50 of Zn, the more Cd was added, the lower was the LC50 value of Zn.

Linear regression demonstrated that LC50 values of Zn decreased significantly upon increasing total Cd concentration ($p < 0.0001$) (Figure 5.3). The linear relationship became more apparent when LC50 values of Zn were expressed on the basis of porewater concentration of Cd or $CaCl_2$-extractable Cd concentration. All the LC50 values of Zn in mixtures were lower than that in the treatments of Zn alone. An increase in the measured total concentration of Cd from 2 mg/kg to 734 mg/kg resulted in a 2.5-fold decrease (1499 to 600 mg/kg of Zn) of the LC50 of Zn for *A. caliginosa.* Up to 3-fold reduction in LC50 values of porewater Zn was observed when the porewater concentration of Cd increased from 31 to 8194 μg/L. An almost 4-fold decrease in LC50 values of $CaCl_2$-extractable Zn was found when the concentration of $CaCl_2$-extractable Cd increased from 1 to 211 mg/kg. These results are consistent with the null hypothesis that Zn toxicity increases with the increasing Cd content.

The joint effects (TU_{mix}) of Cd-Zn were estimated using Equation 5-2 and presented in Figure 5.4. Detailed methods of calculation are shown in Tables S5.3 through S5.5 (Supporting Information). When the measured total concentration was used to express exposure, all values of TU_{mix} of Cd-Zn combinations were above 1. Cadmium addition at any ratio resulted in an antagonistic effect regarding Zn toxicity. TU_{mix} increased from 1.10 to 2.54 with increasing measured total Cd concentration, indicating that more Cd in the mixture resulted in the enhancement of the magnitude of antagonism. TU_{mix} values over the full concentration range decreased to some extent but were still above 1 when the exposures were normalized to porewater or $CaCl_2$-extractable metal concentrations instead of measured total concentrations. There was a tendency towards nearly concentration-addition (TU_{mix} close to 1)

when the concentrations of Cd in the porewater and the $CaCl_2$-extractable Cd were less than 600 µg/L and 20 mg/kg respectively.

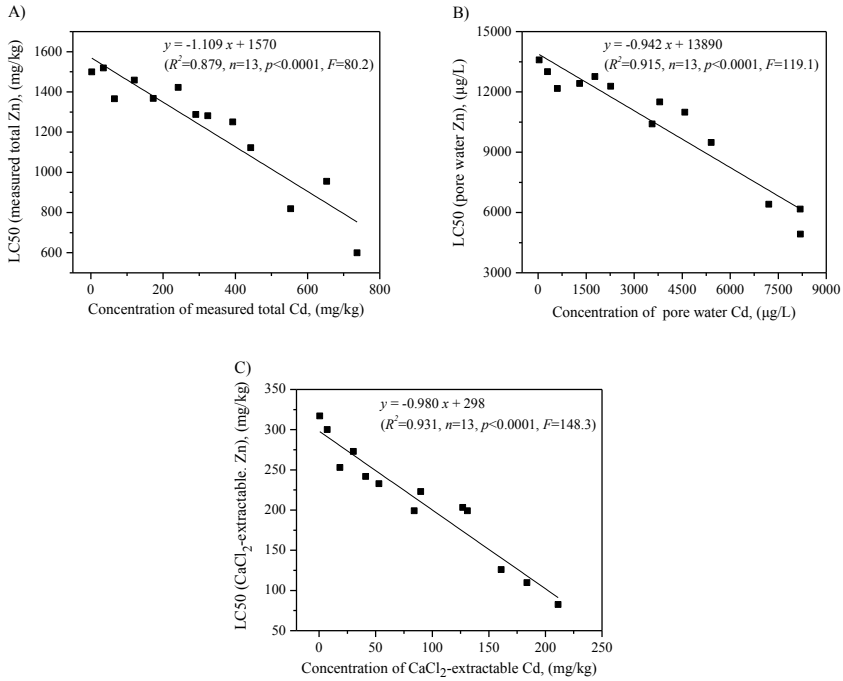

Figure 5.3 Median lethal concentrations (LC50) of Zn for *Aporrectodea caliginosa* under the influence of Cd after 28 d of exposures related to measured total (A), porewater (B) and $CaCl_2$-extractable (C) concentrations. The solid line is the linear fit.

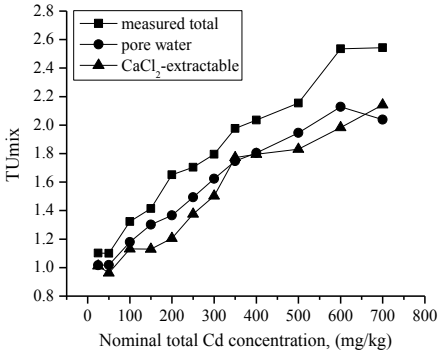

Figure 5.4 Toxic units (TU_{mix}) of Cd-Zn combinations (data points in Figure 5.3) causing 50% mortality of *Aporrectodea caliginosa*. Calculations of TU_{mix} were related to three different exposure metrics (measured total, porewater and $CaCl_2$-extractable concentrations) using equation 5-2.

Impact of Zn on Cd toxicity

A typical observation when establishing dose-response curves was that Zn displayed a protective effect at Cd levels exceeding the LC50 of Cd alone. For instance at a nominal Cd-level of 500 mg/kg, 100% mortality was observed. Subsequent addition of a small amount of Zn alleviated toxicity and induced survival of the earthworms at the same Cd level. Higher concentrations of Zn (nominal total Zn > 600 mg/kg in this example) resulted in mortality again.

Variations in 28 d LC50 values of Cd to *A. caliginosa* at different Zn levels are plotted in Figure 5.5. The LC50 values of Cd varied over a range of Zn concentrations. Firstly, a substantial reduction of Cd toxicity under Zn addition was observed for all three expressions of metal concentrations. When measured total Zn was varied from 100 to 1000 mg/kg, LC50 values of Cd increased from 344 to 680 mg/kg. At these levels, Zn thus exerted a protective effect on Cd toxicity. Secondly, beyond a critical Zn concentration of around 1000 mg/kg for total Zn, 9000 µg/L for porewater Zn or 150 mg/kg for $CaCl_2$-extractable Zn, LC50 values of Cd decreased dramatically with increasing Zn concentration.

The patterns of Zn-Cd interaction reflected by the values of TU_{mix} are demonstrated in Figure 5.6. Calculation procedures are shown in Tables S5.6 through S5.8 (Supporting Information). It was found that TU_{mix} increased from 1.56 to 2.70 upon measured total Zn addition of 183 to 1087 mg/kg, and then reduced slowly to nearly 1 at higher concentrations of measured total Zn. The trends were similar for all three exposure metrics. Nearly all Zn-Cd mixtures showed an antagonistic relationship (TU_{mix} > 1.10) except when 1200 mg/kg of nominal total Zn was added. At this exception, the low LC50 value of Cd indicated that Zn is the dominant cause of mortality in the mixture.

Mixture toxicity modelling

The mixture data of all three exposure metrics were all analyzed. The parameters (*a* and *b*) and significance test results ($p(\chi^2)$) obtained from fitting the nested MIXTOX model are shown in Table 5.4. Generally, the interaction patterns reflected by the modelling were consistent with the results obtained by the calculations of TU_{mix}. In the present study, both the TU approach and MIXTOX modelling showed that the interaction of the metals studied was mainly antagonistic, and the magnitude was both dose ratio- and dose level-dependent.

Fitting of the concentration addition model to the data explained 47% of the variation in the data. Inclusion of parameter *a* in Equation 5-3 to describe synergy or antagonism decreased the sum of the square residuals significantly ($p(\chi^2) = 3.9 \times 10^{-12}$) and explained 57% of the variation in the data. Parameter *a* being positive was an indication of an antagonistic relationship between Cd and Zn. Extending the concentration addition model with a second parameter b_{Zn} to describe a dose ratio-dependent deviation provided the best description of the data and 61% of the variation in the data was explained. Estimated values of the deviation parameters were $a = 3.94$ and $b_{Zn} = -3.99$. It can be concluded from these values that the deviation from additivity was mainly antagonistic. An increased joint effect was connected to a high proportion of Zn in the mixture and a decreased joint effect was related to a high proportion of Cd in the mixture. Similar analysis demonstrated that including a second parameter b_{DL} to describe a dose level dependence also improved the model fit significantly. In this case, parameter *a* being positive and b_{DL} being negative

revealed that the interaction pattern was antagonism and the magnitude of antagonism was dose level dependent.

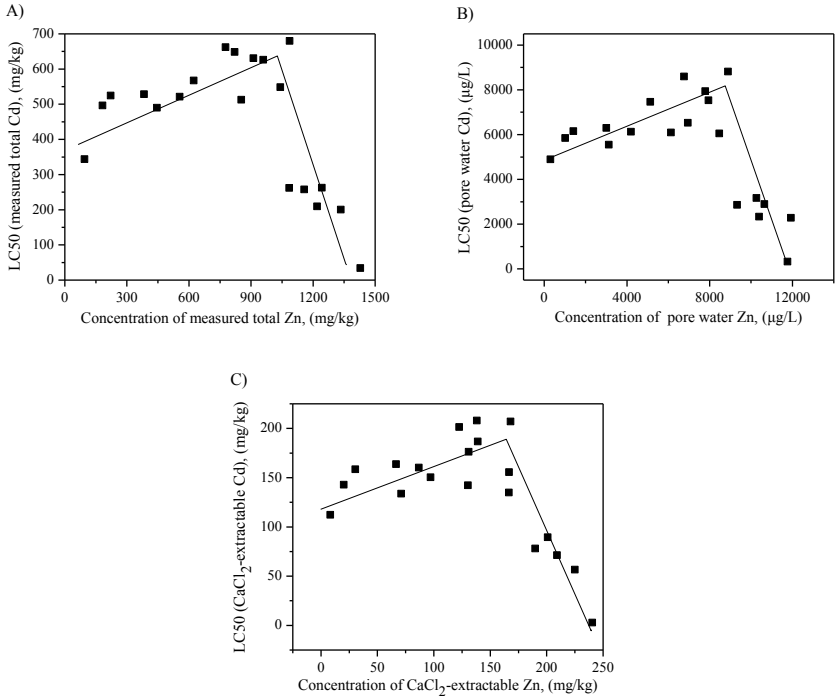

Figure 5.5 Median lethal concentrations (LC50) of Cd for *Aporrectodea caliginosa* under the influence of Zn after 28 d of exposures related to measured total (A), porewater (B) and $CaCl_2$-extractable (C) concentrations. The solid line is freehand and was added to schematically depict trends in mixture toxicity.

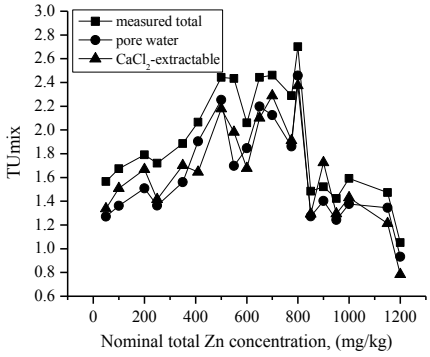

Figure 5.6 Toxic units (TU_{mix}) of Zn-Cd combinations (data points in Figure 5.5) causing 50% mortality of *Aporrectodea caliginosa*. Calculations of TU_{mix} were related to three different exposure metrics (measured total, porewater and $CaCl_2$-extractable concentrations) using equation 5-2.

When relating toxicity to the porewater concentrations or the $CaCl_2$-extractable concentrations, the identified interaction patterns were similar to those of the measured total concentrations.

Table 5.4 Parameter values of the deviation function and significance

	R^{2*}	a	b_{Zn}/b_{DL}	$p(\chi^2)$
Measured total				
CA**	0.47	NA	NA	NA
S/A	0.57	1.62	NA	3.9×10^{-12}
DR	0.61	3.94	-3.99	1.2×10^{-15}
DL	0.59	0.04	-23.82	3.5×10^{-13}
Porewater				
CA	0.48	NA	NA	NA
S/A	0.54	1.86	NA	8.6×10^{-8}
DR	0.59	4.27	-4.68	6.5×10^{-12}
DL	0.55	0.05	-22.96	4.8×10^{-8}
$CaCl_2$-extractable				
CA	0.52	NA	NA	NA
S/A	0.54	1.02	NA	5.1×10^{-4}
DR	0.58	3.67	-4.79	9.1×10^{-8}
DL	0.55	0.02	-42.17	5.3×10^{-4}

*The value R^2 quantifies goodness of fit. Parameters (a, b_{Zn}/b_{DL}) define the deviation patterns from concentration additive model, and $p(\chi^2)$ indicates the statistical outcome of the Chi-squared test. **CA indicates concentration addition; S/A indicates synergy/antagonism; DR indicates dose ratio dependent; DL indicates dose level dependent. NA indicates not applicable.

5.4 Discussion

A number of studies have examined the toxicity of Cd and Zn presented singly for selected life-cycle traits in earthworms (Fitzpatrick et al., 1996; Khalil et al., 1996; Spurgeon et al., 2000). Although variations in soil types, exposure time and species sensitivity all contributed to the variety of metal toxicity reported, the LC50 values of individual Cd and Zn in this study were still comparable to data in some literature (Fitzpatrick et al., 1996; Spurgeon et al., 2000). The effect of a mixture of Cd and Zn on the mortality of *A. caliginosa* was mainly antagonistic regardless of how the exposure conditions were measured. Previous studies revealed that the most frequently observed pattern of interaction in Cd and Zn mixtures was antagonism, followed by additivity (Khalil et al., 1996; Van Gestel and Hensbergen, 1997; Weltje, 1998). Possible interactions between Cd and Zn limited the predictive capability of the simple concentration-addition model. Mixture toxicity was a result of the simultaneous action at all interaction levels. This study focused on the interaction at the exposure level and at the toxicological level. The relative contributions of interactions outside the organism and inside the organism to toxic effects were then distinguished.

Partition coefficients of each component in the mixtures were predictive for metal interactions occurring in the soil. The average K_d value of metals in the present study indicated that the order of sorption affinity was Zn > Cd. Empirically, the metal with higher K_d value affected partitioning of the metal with lower K_d (Jonker et al., 2004). Here, however, the partitioning of Cd was largely unaffected by the presence of Zn, and vice versa. Apparently, sufficient soil sorption sites are available to preclude competition of Cd and Zn for sorption to the solid phase, as evidenced by the fairly constant K_d values found even at highly elevated Cd or Zn concentrations. Hence, there was no interaction of Cd and Zn at the exposure level. In a study carried out by Van Gestel and Hensbergen (1997), Cd did not affect the water-soluble concentration of Zn in an artificial soil substrate, in agreement with our results. On the other hand, water extractability of Cd was significantly increased by Zn (p = 0.003). These authors attributed this to increased complexation of Cd with chloride, which was less important for Zn. There was no chloride complexation in the soil we studied as the metals were spiked as their acetate salts. The addition of Cd alone and Zn alone and as a mixture did not cause a dose-related decrease of pH, and only minor fluctuations in pH values. This indicated that after equilibration for two months, the acetate had been mineralized. Zidar et al. (2009) found that water extractability of Cd in artificially contaminated food for the isopod *Porcellio scaber* was not significantly affected by the presence of Zn at any concentration, and vice versa ($p > 0.05$). Since metals were added as nitrates, they concluded that the organic food source, high in organic matter, probably accounted for the non-interaction between Cd and Zn at the exposure level.

Unraveling the role of interactions in soil requires further analysis of the relationship between toxic effects and metal concentrations occurring in different phases and species (Jonker et al., 2004). It is generally agreed that exposure is governed mainly by the solution phase (porewater), and the toxicity of metals is dependent on the nature of the metal species present (Peijnenburg et al., 2007; Peijnenburg and Jager, 2003; Saxe et al., 2001). Porewater and $CaCl_2$-extractable concentrations were often used to express exposure and were assumed to be available for uptake (Janssen et al., 1997; Kalis et al., 2008). When examining the combined toxicity of simple mixtures, Jonker et al. (2004) found that the Freundlich adsorption constant of one metal was influenced by the presence of another metal, suggesting interaction at the exposure level. As a result, both the composition of the mixture and the relative toxicity of each mixture component differed between total, water-soluble, and $CaCl_2$-extractable concentrations. In this experiment, relative consistent modes of interaction were observed when LC50s were expressed as total, porewater and $CaCl_2$-extractable metal concentrations. One comment should be made with regard to the different expressions of exposure yielding similar results. It may be due partially to our use of only one soil type. Total metal concentrations correlated well with the porewater and $CaCl_2$-extractable concentrations. As the bioavailability of metals can vary over several orders of magnitude depending on the soil properties, it is probably critical to include more soil types to study the interaction patterns of metal mixtures.

Metal toxicity involves three steps: bioavailable metal causes exposure; exposure leads to uptake; and effects result from reaction with a biological target. In *A. caliginosa* Cd and Zn interacted at the effect level. Addition of Cd induced higher Zn toxicity. Already at a low

concentration (measured total Cd = 65 mg/kg), Cd antagonized Zn toxicity. When the primary constituent was Cd, addition of Zn reduced toxicity.

Because competitive displacement between Cd and Zn did not occur in the soil we studied, we examined whether interactions could be identified during uptake and at the effect level. Competition between Cd and Zn during the uptake processes has been reported in plants (Cataldo et al., 1983). It is likely that Cd and Zn share the same pathway of uptake since they both belong to the group IIB of the periodic table and have similar physicochemical properties. Nevertheless, Li et al. (2010) provided evidence that the uptake of Cd by *Eisenia fetida* proceeds via Ca channels whereas Zn uptake is carrier-mediated by proteins or other sulphydryl-containing compounds, implying that the mechanisms of Cd and Zn uptake in *Eisenia fetida* are essentially different. Therefore, Cd and Zn may employ different mechanisms in influencing the uptake of each other.

In addition, interactions at the internal pathways may happen, such as the synthesis of specific metal-binding proteins induced by another metal. Metallothionein (Mt), a metal-binding protein, plays an important role in preventing toxicity from heavy metals (e.g., Cd) by formation of metal-Mt compounds (Brzóska and Moniuszko-Jakoniuk, 2001). Metallothionein induced by Zn pretreatment or co-administration can immobilize Cd within the earthworms, which at least in part accounts for the protective action of Zn supply against Cd toxicity. Barata et al. (2006) mentioned that since both Cd and Zn can activate Mt synthesis, there is a high probability of competition with Mt compounds and that for this reason their combined effects may be expected to be antagonistic.

Different from traditional viewpoints, Wang et al. (2011b) argued that the actual toxic effect may not be due to interactions of the metals at binding sites. Their study showed that rather than site-specific competition, electrostatic interactions at the cell membrane surface of organisms may account for the alleviation of toxicity. It should be noted that all of the above assumptions of interaction at various uptake pathways or bindings sites only serve as examples of possible mechanisms in which interaction of the metals of interest could occur. More attention should be paid to the characterization of toxicokinetics and toxicodynamic of metals and target site interactions in future studies in order to obtain reliable interaction mechanisms and provide a robust biological basis for the observed differences.

After rescaling the concentrations in terms of toxic units, interactions between metals were obtained. When focusing on the impact of Cd on Zn toxicity, antagonism was noticed in nearly all cases. Since the addition of Cd linearly increased Zn toxicity, it was expected that the interaction type is synergy. On a molar basis, Cd is about 7 times more toxic than Zn. Therefore, the replacement of Zn by Cd in action sites may produce a larger adverse effect than Zn-only treatments. It is difficult to explain the reason why synergy was not observed. When focusing on the impact of Zn on Cd toxicity, it was found that Zn exerted a beneficial action on Cd toxicity when it was administrated at a measured total concentration in between 0 and 1000 mg/kg. Calculations of TU_{mix} revealed that the antagonistic effect of Cd and Zn coincided with the protective effect of Zn. The decreasing effects of Cd with increasing concentrations of Zn were described previously for the house cricket *Acheta domesticus* and the beetle *Tenebrio molitor* (Migula et al., 1989). The amount of Zn added is critical in determining whether a protective or harmful effect is exerted by Zn. When the total Zn

concentration was beyond 1000 mg/kg in this study, the LC50 of Cd rapidly decreases. Excess Zn thus induced a high toxicity and masked the toxicity of Cd.

In addition to toxic unit approach, the MIXTOX model approach revealed more complex deviation patterns. It is not easy to compare our results with those of others studies, because of the scarcity of exactly similar species and binary metal combinations. Jonker et al. (2005) analyzed the effect of Cd and Zn on reproduction of the springtail *F. candida*, a dose ratio-dependent deviation from concentration addition was found. Interaction pattern shifted from antagonism to synergy when Zn concentration was 13 times higher than Cd in the mixtures. In the present study, the magnitude of antagonism decreased with increasing concentration ratios of Zn to Cd in the mixtures. Besides, a concentration-dependent joint effect was also found. Lock and Janssen (2002) studied mixture toxicity of Cd and Zn to the potworm *Enchytraeus albidus* and found that the effect predictions based on the concentration addition model were always higher than those obtained with the independent action model. Therefore, it is reasonable to base results of this study on concentration addition model, which represents a worst-case scenario for risk assessment of mixture toxicity.

The interaction pattern can also vary when using different endpoints (Van Gestel and Hensbergen, 1997) and different expressions of exposure (Weltje, 1998). The combined effect of Cd and Zn on the growth of the springtail *F. candida* was antagonistic (EC50 significantly higher than a TU of 1.0), while the effect on reproduction was additive (EC50 not significantly different from a TU of 1.0) (Van Gestel and Hensbergen, 1997). Weltje (1998) compiled data on sublethal toxicity and tissue concentrations of Cd, Cu, Pb and Zn mixtures in earthworms. Mixture toxicity shifted from mainly antagonism towards nearly concentration-addition when the endpoints were based on extractable metal concentrations instead of total soil concentrations. Zidar et al. (2009) reported that effects of Cd and Zn on food consumption by the isopod *Porcellio scaber* were additive when based on total and water-soluble concentration but antagonistic when related to tissue concentrations. In this study, the toxic effect was estimated only based on mortality, so no conclusion on the impact of different endpoints could be drawn. There was also no observed shift in response types when expressing exposure using bioavailable concentrations instead of total concentrations.

5.5 Conclusions

This study provided insight into the interactive effect of binary Cd and Zn mixtures on mortality of *A. caliginosa* at different interaction levels in one soil type. Toxicity of Cd and Zn mixtures could not be predicted on the basis of the individual metals. Simultaneous exposure to Cd and Zn in soil jointly affected earthworm mortality, but did not influence their partitioning between solid and solution phases. Interactions of both Cd-Zn and Zn-Cd were mainly antagonistic as shown by the values of TU_{mix}. Using the MIXTOX model, deviation from additivity was statistically quantified to be mainly antagonistic, and the magnitude of antagonism depended on both relative concentrations and concentration magnitudes for the whole ranges of concentrations tested. Metal-metal interactions most likely occurred at the organism level.

Supporting Information

Table S5.1 Selected physicochemical properties of the tested soil.

Soil properties	Values
pH	6.1 ± 0.3*
Texture	sandy loam
Clay content (%)	7.0 ± 0.6
Organic matter (%)	5.7 ± 0.2
CEC (cmol/kg)	10.1 ± 0.3
Metal content	
Total Zn (mg/kg, dw)	104 ± 10.2
Total Cd (mg/kg, dw)	1.43 ± 0.37

*All values are presented as mean ± standard deviation for three replicates.

Table S5.2 Single metal (Cd, Zn) toxicity for *Aporrectodea caliginosa* after 28 d of exposure related to nominal, measured total, porewater and $CaCl_2$-extractable concentrations.

	Nominal concentration (mg/kg)	Measure total concentration (mg/kg)	Porewater concentration (mg/kg)	$CaCl_2$-extractable concentration (mg/kg)	Mortality (%)
Cd	0	0	0	0	0
	50	75	993	24	0
	100	115	950	41	0
	150	170	1425	62	0
	200	207	2715	78	0
	250	251	3175	89	0
	300	301	3830	97	0
	350	348	4970	114	75
	500	514	9085	193	100
	650	737	10700	282	100
	800	754	7574	322	100
	1000	919	13050	361	100
Zn	0	0	0	0	0
	50	228	585	34	0
	100	270	2188	63	0
	200	333	4678	85	0
	410	812	6360	133	0
	600	818	7022	170	25
	650	902	7495	180	0
	775	909	8650	205	0

850	1053	10065	219	0
950	1033	10209	239	0
1200	1295	12350	288	0
1300	1461	13183	307	25
1350	1500	13594	317	50
1400	1538	14003	327	75
1500	1815	14813	347	100
2000	2252	18728	395	100

Table S5.3 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Cd-Zn combinations used here are corresponding to data points of Figure 5.3A).

Nominal Cd (mg/kg)	Cd-Zn combinations causing 50% mortality of earthworms		TU_{Cd}*	TU_{Zn}	TU_{mix} (TU_{Cd}+TU_{Zn})	Interaction type
	Measured total Cd, (mg/kg)	LC50 (measured total Zn), (mg/kg)				
0	2	1499	-	-	-	-
25	35	1500	0.10	1.00	1.10	Antagonistic
50	65	1366	0.19	0.91	1.10	Antagonistic
100	120	1459	0.35	0.97	1.32	Antagonistic
150	173	1368	0.50	0.91	1.41	Antagonistic
200	242	1422	0.70	0.95	1.65	Antagonistic
250	291	1288	0.84	0.86	1.70	Antagonistic
300	324	1281	0.94	0.85	1.79	Antagonistic
350	393	1250	1.14	0.83	1.97	Antagonistic
400	443	1122	1.29	0.75	2.04	Antagonistic
500	553	818	1.61	0.55	2.16	Antagonistic
600	653	955	1.90	0.64	2.54	Antagonistic
700	737	600	2.14	0.40	2.54	Antagonistic

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing measured total Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.4 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Cd-Zn combinations used here are corresponding to data points of Figure 5.3B).

Nominal Cd (mg/kg)	Cd-Zn combinations causing 50% mortality of earthworms		TU_{Cd} *	TU_{Zn}	TU_{mix} (TU_{Cd}+TU_{Zn})	Interaction type
	Porewater Cd, (µg/L)	LC50 (porewater Zn), (µg/L)				
0	31	13588	-	-	-	-
25	295	13000	0.06	0.96	1.02	Additive
50	601	12163	0.12	0.90	1.02	Additive
100	1299	12417	0.27	0.91	1.18	Antagonistic
150	1774	12764	0.36	0.94	1.30	Antagonistic
200	2269	12274	0.46	0.90	1.36	Antagonistic
250	3562	10399	0.73	0.77	1.50	Antagonistic
300	3803	11503	0.78	0.85	1.63	Antagonistic
350	4586	10990	0.94	0.81	1.75	Antagonistic
400	5408	9484	1.11	0.70	1.81	Antagonistic
500	7209	6412	1.47	0.47	1.94	Antagonistic
600	8186	6166	1.67	0.45	2.12	Antagonistic
700	8194	4920	1.68	0.36	2.04	Antagonistic

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing porewater Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.5 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Cd-Zn combinations used here are corresponding to data points of Figure 5.3C).

Nominal Cd (mg/kg)	Cd-Zn combinations causing 50% mortality of earthworms		TU_{Cd}*	TU_{Zn}	TU_{mix} (TU_{Cd}+TU_{Zn})	Interaction type
	$CaCl_2$-extr. Cd, (mg/kg)	LC50 ($CaCl_2$-extr. Zn), (mg/kg)				
0	1	317	-	-	-	-
25	7	300	0.06	0.95	1.01	Additive
50	18	253	0.16	0.80	0.96	Additive
100	30	273	0.27	0.86	1.13	Antagonistic
150	41	242	0.37	0.76	1.13	Antagonistic
200	53	233	0.47	0.73	1.20	Antagonistic
250	84	199	0.75	0.63	1.38	Antagonistic
300	90	223	0.80	0.70	1.50	Antagonistic
350	127	203	1.13	0.64	1.77	Antagonistic
400	131	199	1.17	0.63	1.80	Antagonistic
500	161	126	1.43	0.40	1.83	Antagonistic
600	184	110	1.64	0.35	1.99	Antagonistic
700	211	83	1.88	0.26	2.14	Antagonistic

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing $CaCl_2$-extractable Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.6 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Zn-Cd combinations used here are corresponding to data points of Figure 5.5A).

Nominal Zn (mg/kg)	Zn-Cd combinations causing 50% mortality of earthworms		TU_{Cd} *	TU_{Zn}	TU_{mix} (TU_{Cd}+TU_{Zn})	Interaction type
	LC50 (measured total Cd), (mg/kg)	Measured total Zn, (mg/kg)				
0	344	95	-	-	-	-
50	497	183	1.44	0.12	1.56	Antagonistic
100	525	222	1.53	0.15	1.68	Antagonistic
200	528	383	1.54	0.26	1.80	Antagonistic
250	490	445	1.42	0.30	1.72	Antagonistic
350	521	555	1.52	0.37	1.89	Antagonistic
410	568	623	1.65	0.42	2.07	Antagonistic
500	662	777	1.93	0.52	2.45	Antagonistic
550	649	821	1.89	0.55	2.44	Antagonistic
600	513	853	1.49	0.57	2.06	Antagonistic
650	631	911	1.83	0.61	2.44	Antagonistic
700	627	959	1.82	0.64	2.46	Antagonistic
775	548	1042	1.59	0.69	2.28	Antagonistic
800	680	1087	1.98	0.72	2.70	Antagonistic
850	262	1085	0.76	0.72	1.48	Antagonistic
900	258	1157	0.75	0.77	1.52	Antagonistic
950	209	1220	0.61	0.81	1.42	Antagonistic
1000	262	1243	0.76	0.83	1.59	Antagonistic
1150	200	1334	0.58	0.89	1.47	Antagonistic
1200	34	1429	0.10	0.95	1.05	Additive

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing measured total Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.7 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Zn-Cd combinations used here are corresponding to data points of Figure 5.5B).

Nominal Zn (mg/kg)	Zn-Cd combinations causing 50% mortality of earthworms		TU_{Cd}*	TU_{Zn}	TUmix (TU_{Cd}+TU_{Zn})	Interaction type
	LC50 (porewater Cd), (µg/L)	Porewater Zn, (µg/L)				
0	4890	303	-	-	-	-
50	5848	1020	1.20	0.08	1.28	Antagonistic
100	6152	1406	1.26	0.10	1.36	Antagonistic
200	6295	3001	1.29	0.22	1.51	Antagonistic
250	5546	3120	1.13	0.23	1.36	Antagonistic
350	6124	4198	1.25	0.31	1.56	Antagonistic
410	7464	5133	1.53	0.38	1.91	Antagonistic
500	8590	6760	1.76	0.50	2.26	Antagonistic
550	6095	6129	1.25	0.45	1.70	Antagonistic
600	6531	6946	1.34	0.51	1.85	Antagonistic
650	7943	7786	1.62	0.57	2.19	Antagonistic
700	7534	7945	1.54	0.58	2.12	Antagonistic
775	6053	8469	1.24	0.62	1.86	Antagonistic
800	8819	8890	1.80	0.65	2.45	Antagonistic
850	2864	9325	0.59	0.69	1.28	Antagonistic
900	3169	10261	0.65	0.76	1.41	Antagonistic
950	2333	10390	0.48	0.76	1.24	Antagonistic
1000	2897	10645	0.59	0.78	1.37	Antagonistic
1150	2286	11929	0.47	0.88	1.35	Antagonistic
1200	325	11765	0.07	0.87	0.94	Additive

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing porewater Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Table S5.8 Toxic units of binary mixtures of Cd and Zn, and the identified interaction types (Zn-Cd combinations used here are corresponding to data points of Figure 5.5C).

Nominal Zn (mg/kg)	Zn-Cd combinations causing 50% mortality of earthworms		TU_{Cd}*	TU_{Zn}	TUmix (TU_{Cd}+TU_{Zn})	Interaction type
	LC50 ($CaCl_2$-extr. Cd), (mg/kg)	$CaCl_2$-extr. Zn, (mg/kg)				
0	112	8	-	-	-	-
50	143	20	1.27	0.06	1.33	Antagonistic
100	158	31	1.41	0.10	1.51	Antagonistic
200	164	67	1.46	0.21	1.67	Antagonistic
250	134	71	1.19	0.22	1.41	Antagonistic
350	160	87	1.43	0.27	1.70	Antagonistic
410	150	97	1.34	0.31	1.65	Antagonistic
500	201	122	1.79	0.39	2.18	Antagonistic
550	176	131	1.57	0.41	1.98	Antagonistic
600	142	130	1.27	0.41	1.68	Antagonistic
650	187	139	1.66	0.44	2.10	Antagonistic
700	208	138	1.85	0.44	2.29	Antagonistic
775	156	167	1.39	0.53	1.92	Antagonistic
800	207	168	1.84	0.53	2.37	Antagonistic
850	78	190	0.69	0.60	1.29	Antagonistic
900	135	167	1.20	0.52	1.73	Antagonistic
950	71	209	0.64	0.66	1.30	Antagonistic
1000	90	201	0.80	0.63	1.43	Antagonistic
1150	57	225	0.50	0.71	1.21	Antagonistic
1200	3	240	0.03	0.76	0.79	Synergistic

*Toxic unit of Cd (TU_{Cd}) was calculated by dividing $CaCl_2$-extractable Cd concentration in mixture to median lethal concentration (LC50) of Cd presented singly. Mixture toxic units (TU_{mix}) are the sum of toxic units of Cd (TU_{Cd}) and Zn (TU_{Zn}).

Chapter VI

General Discussion

A comprehensive understanding of the mechanisms of toxicity is needed in order to effectively predict metal effects on soil organisms. In this PhD thesis, we investigated the responses of different earthworm species to single metals (Cu, Ni, Cd, and Zn) and a binary mixture of Cd and Zn in metal-spiked soils. These soil toxicity data allow to account explicitly for the role of bioavailability, ecophysiological factors and mixture interactions in determining metal toxicity.

Our research was based upon four research questions, which are answered in the preceding four chapters:

[1] Which cations (H^+, Ca^{2+}, Mg^{2+}, K^+ and Na^+) exert significant effects on metal toxicity and how could these toxicity-modifying factors be incorporated into terrestrial toxicity models developed on the basis of the BLM theory? (Chapters II and III)

[2] Are the toxicity-modifying factors the same for different earthworm species (*Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*) and for different metals (Cu, Cd, and Ni)? (Chapters II and III)

[3] Are metal (Cu, Cd, Ni, and Zn) accumulation pattern and sensitivity of earthworms species-specific? Can species-specific traits of earthworms provide a clue for predicting metal accumulation and toxicity? (Chapter IV)

[4] Where do the interactions of mixture components (Cd and Zn) possibly occur and how do they impact the observed toxicity? (Chapter V).

The biotic ligand model is often used as the state-of-the-art approach to quantify the link between chemical availability and metal toxicity in aquatic systems. It is a synthesis of decades of work on metal speciation, accumulation, toxicity and physiology (Paquin et al., 2002). In this thesis, we tried to apply the BLM theory to soil organisms, with a special focus on earthworms. The possible dependence of metal accumulation and toxicity on species-specific traits was investigated. Integration of effect models and traits-based approaches may offer a means to facilitate extrapolation of accumulation and toxicity data cross species. We also distinguished the mixture interactions at different levels and determined the impact of mixture interactions on the observed toxicity. Findings in this PhD thesis open the perspective of developing a mechanistic framework that is generally applicable to predict the effects of metals and metal mixtures on soil organisms.

This concluding chapter provides a synthesis of the preceding four separate chapters, which have been published as independent papers in peer-reviewed journals. Prior to an integrated discussion and a future outlook, answers to the research questions are provided below.

6.1 Answers to research questions

Research question 1: Only H^+ not other cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) exerted a significant role in alleviating Cu toxicity to earthworms (Chapter II). Mg^{2+} was identified as the sole factor significantly alleviating Ni toxicity, while no significant influence of cations (H^+, Ca^{2+}, Mg^{2+}, K^+ and Na^+) on Cd toxicity to earthworms was observed (Chapter III). A multicomponent Freundlich model, complying with the basic assumptions of BLM theory, was used to link Cu toxicity to free Cu^{2+} activity and possible protective cations (i.e., H^+) in soil porewater (Chapter II). The Freundlich-type model in which the protective effects of H^+ were incorporated, accounted for 84% to 96% of the variation in LC50{Cu^{2+}} for earthworms. Based on empirical studies and BLM theory, the free ion approach was proposed to quantify Ni and Cd toxicity (Chapter III). By incorporating the competitive effects of Mg^{2+} into the free ion approach, more than 84% of the variation in LC50{Ni^{2+}} for earthworms was explained. The free ion activity model, which is a special case of the free ion approach with no impact of cations, sufficiently described the variability in LC50{Cd^{2+}}.

Research question 2: The same toxicity-modifying factor (pH) was identified for Cu toxicity to the three tested earthworm species *L. rubellus*, *A. longa* and *E. fetida* (Chapter II). External validation also showed a pH dependence of Cu toxicity for other soil organisms with different endpoints (*E. fetida* cocoon production, *Folsomia candida* juvenile production, *Hordeum vulgare* root elongation, *Lycopersicon esculentum* shoot yield, glucose induced respiration, and potential nitrification rate). The same factor (Mg^{2+}) was found to significantly modify Ni toxicity to the two tested earthworm species *L. rubellus* and *A. longa*. No significant toxicity-modifying factor was observed for Cd toxicity to *L. rubellus* and *A. longa* (Chapter III). There was a lack of consistent effects from the presence of possible competing cations for different metals. Based on these findings it is concluded that metal toxicity to earthworms needs to be evaluated on a metal-specific basis.

Research question 3 (Chapter IV): Accumulation and toxicity of Cu, Cd, Ni, and Zn in the earthworms *L. rubellus*, *A. longa* and *E. fetida* were species-specific. At the same exposure concentration, internal concentrations followed the order: *L. rubellus* > *E. fetida* > *A. longa* for Cu and Ni, *L. rubellus* ≈ *E. fetida* ≈ *A. longa* for Cd, and *L. rubellus* > *A. longa* > *E. fetida* for Zn. The concentrations of Cu, Cd, and Zn in *E. fetida* generally levelled off at high exposure concentrations but not for the other two species. *A. longa* showed a high capability of regulating internal Ni concentrations. Based on traits theory, a set of traits (such as soil habitat, mobility, ratio of surface area to mass) can be related to the metal accumulation ranking of the earthworm species tested. However, implementing the traits theory for quantitatively explaining all the observed accumulation pattern of different earthworm species was shown to be difficult. For all the four metals tested *L. rubellus* was the most sensitive species, followed by *A. longa* and E. *fetida*. Differences in sensitivity between species might be explained using the following traits: soil habitat, calcium glands activity, ratio of surface area to mass, and immune-competent cells. While, ranking of species sensitivity cannot be quantitatively linked to one specific trait. The traits-based approaches suggested that most likely a group of earthworm traits together determined (differences in) metal accumulation and sensitivity.

Research question 4 (Chapter V): The interactions between Cd and Zn in soil (at the exposure level) were estimated by comparing partition coefficients of one metal in the

presence and absence of the second metal. The partitioning of Cd and Zn between soil solid phase and porewater was affected neither by the concentrations nor by the presence of the other metal, suggesting no interactions at the exposure level. Simultaneous exposure to Cd and Zn in soil jointly affected the mortality of the earthworm *A. caliginosa*. Increasing Cd concentration decreased Zn toxicity. The addition of Zn over a certain range (100 to 1000 mg/kg) decreased Cd toxicity. Beyond a critical Zn concentration of around 1000 mg/kg, Cd toxicity dramatically increased. Interactions of Cd and Zn occurred at the organism level, limiting the predictive ability of the simple concentration addition model. MIXTOX modelling showed that mixture interactions were mainly antagonistic and the magnitude of antagonism depended on both the relative concentrations of Cd and Zn and the concentration magnitudes for the whole ranges of concentrations tested. These findings highlight the importance of identifying the relative influence of various interactions from external exposure to internal assimilation in assessing metal toxicity.

6.2 Applicability of BLM theory in soil

The results in this thesis provide evidence in favour of the applicability of BLM theory to soil organisms, especially earthworms, exposed in a range of metal-spiked soils of varying properties (Chapter II and III). Applying the BLM theory to soil toxicity data has rarely been done before. Our research therefore added new knowledge and provided alternative tools with regard to the explanation and prediction of the variability in Cu, Cd, and Ni toxicity across soils.

The BLMs for aquatic organisms have been well developed and scientifically understood (Niyogi and Wood et al., 2004; Paquin et al., 2002; Santore et al., 2002). Some of them have even been incorporated into water quality regulations, for example, the US Environmental Protection Agency's aquatic life freshwater quality criteria for copper (USEPA, 2007). And, in the EU risk assessment reports, the BLMs for chronic toxicity of Cu, Ni, and Zn have also been recommended for regulatory use (Denmark, 2008; ECI, 2008; The Netherlands, 2008). A major strength of the BLM is that it provides an operational framework for modelling bioavailability and toxicity under various environmental conditions. Because of assumed similarity in toxicity mechanisms between aquatic and terrestrial organisms (as explained in paragraph 1.3 of Chapter I), it has been postulated that the BLM can be applied to terrestrial organisms. In this thesis, we therefore aimed to expand the scope of BLM beyond the initial emphasis on metal toxicity in the aquatic environment to metal toxicity in the soil environment. However, the complexity of the soil system presents a barrier of developing biotic ligand models for soil organisms exposed in real soils.

The routes of metal uptake in soils are generally more complex than those in solution systems. It remains problematic to determine the relative importance of each exposure pathway (porewater, ingestion of food and soil particles) for different soil organisms (Steenbergen et al., 2005). The morphological (e.g., structure of the skin), physiological (e.g., mode of uptake of water and oxygen), and behavioral properties (e.g., food choice) of organisms are important variables in this respect. Uptake via the skin is the most important route of uptake of (pore)water and oxygen for earthworms and other soft-bodied organisms (e.g., enchytraeids, nematodes), while hard-bodied organisms (e.g., arthropods) rely for

uptake of (pore)water and oxygen on specialized organs (Peijnenburg et al., 2012). This has consequences for the validity of the porewater hypothesis, which is a premise for applying BLM theory in soils. In our study, the Freundlich-type model and the free ion approach, complying with the basic assumptions of the BLM, well described metal toxicity to earthworms with dermal uptake of porewater as the main exposure route. However, these models may not be valid for other soil organisms with distinct exposure routes. The BLM theory in soil is more difficult to prove for soil microorganisms. When extrapolating our models to two microbial processes (glucose induced respiration and potential nitrification rate), the model performances were generally weaker than those for soil invertebrates and plants (Chapter II). Soil microorganisms are usually attached to soil particles, implying that the local composition of the microbe bathing solution may differ from soil porewater that can be sampled (Ore et al., 2010). Mertens et al. (2007) developed a model based on BLM theory to predict Zn toxicity to nitrification by *Nitrosospira* sp. in soil and found that there were up to 4 times over- and under-prediction. The main obstacle in evaluating metal bioavailability and toxicity to soil microorganism lies in the difficulty in defining the correct solution to which microorganisms are exposed (i.e., from which pool microorganisms take up metals). Therefore, before explaining and predicting metal toxicity with the BLM theory, uptake routes of the test organism need to be understood.

Besides, unlike simple solution systems it is difficult to univariately manipulate the composition of soil porewater and to separate the effects of each cation on toxicity. Challenges are met in estimating reliable and representative BLM parameters from soil toxicity data. To overcome this, previous attempts to model metal toxicity to soil organisms were often done in hydroponic solutions. But the problem is that model parameters fitted from solution cultures do not match one on one with soil cultures. In this thesis we focused on developing alternative bioavailability modes in order to facilitate the application of BLM theory in soils. The developed Freundlich-type model and the free ion approach, which share the same theoretical basis, allow us to incorporate metal speciation and interactions between metal ions and the possible competing ions in estimating metal toxicity. When applying the BLM directly to soil toxicity data, Thakali and co-workers (2006a; 2006b) found that it is necessary to empirically fix some parameters (e.g., f_{50}) before fitting the binding constants for each cation. The effectiveness, success, and regulatory relevance of BLM benefit from its mechanistic basis, which will suffer if BLM development becomes too much a data-fitting exercise based on faith that simple implementations of the approach are sufficient (Erickson, 2013). The multicomponent Freundlich model or the free ion approach avoids the need to fit separate binding constants for the metal ion and potentially competing cations as the parameters α (n_H/n_M) and β_i (n_{Xi}/n_M) express the binding affinities of H^+ and other cations relative to metal ions. This indicates that these two models require fewer parameters than the common BLM. In the BLM, biotic ligands are considered to be independent and homogeneously distributed, most often represented by a single ligand-binding constant (Slaveykova and Wilkinson, 2005), while the Freundlich-type model considers site heterogeneity. The biological surfaces usually contain multiple sites including physiologically active sites (i.e., biotic ligands or transport sites) and nonspecific, physiologically inactive sites (e.g., cell wall polysaccharides, fish mucus) (Van Leeuwen and Pinheiro, 2001). Several studies have shown the presence of multiple adsorptive sites (Plette

et al., 1996) and multiple metal internalization routes (Hassler and Wilkinson, 2003; Sunda and Huntsman, 1998; Taylor et al., 2000). For instance, two different types of Cu-binding sites (high-affinity, low-capacity sites and low-affinity, high-capacity sites) have been identified on the gills of rainbow trout (Taylor et al., 2000). Therefore, site heterogeneity is expected to be important for metal toxicity. Given these considerations, the Freundlich-based models do have some conceptual and practical advantages over the BLM.

The LC50s for earthworms predicted by the Freundlich-type model or the free ion approach both were generally within a factor of two of the observed values. External validation of the model showed a similar level of precision, even though toxicity data for different soil organisms and for different endpoints were used. A factor of two is an accuracy that is commonly used in risk assessment (Van Leeuwen and Hermens, 2007). Moreover, this is the accuracy that is typically obtained due to biological variability when an ecotoxicology experiment is repeated (Jager, 2013). Our findings therefore support the applicability of BLM theory for terrestrial species exposed in soil. In spite of this accurate prediction, there were shortcomings to the models. Statistical analysis revealed a strong correlation between a fair amount of porewater parameters (Chapter III). In other words, the possible effect of each individual cation cannot be isolated and verified directly. The evident ameliorative effects of cations (e.g., Mg^{2+} on Ni^{2+}) are possibly due to the covariance of their activities with Ni^{2+} activities rather than their competitive binding at the biotic ligand sites. Here, stepwise multiple linear regression analysis and the Akaike information criterion were used to identify the variables significantly determining toxicity whilst at the same time balancing the complexity and the goodness-of-fit of the model. An important implication of our studies is that the most useful approach to model and predict effects of metals on organisms will likely involve trade-offs among the predictive ability required, porewater parameters and the cost to obtain them.

For the purpose of mechanistic studies, a solution system is often used in the toxicity test to provide direct evidence for the protective effects of cations by univariately controlling the composition of the test solutions. Previous studies with soil invertebrates and plants exposed in simple solution systems show that the application of the BLM to terrestrial organisms is theoretically and empirically feasible (Steenbergen et al., 2005; Li et al., 2008; Lock et al., 2007). This kind of research therefore lends credence to the theory behind our terrestrial models. Ideally, the results obtained in the solution system can be qualitatively or quantitatively extrapolated to natural soils. For qualitative validation, it needs to be checked whether the identified toxicity-modifying factors in solution cultures are also significantly affecting metal toxicity to organisms in soil cultures. For quantitative validation, it needs to be checked whether the same BLM parameters (binding constants and f_{50}) hold for the tested species in soil. In reality, extrapolation of the developed BLMs from solution to soil systems is generally difficult as the geochemistry of solution systems often bears little resemblance to those of real world soils (He et al., 2014). A structural underestimation of metal toxicity was found when directly applying the BLM developed from solution-sand system to soils (Koster et al., 2006). This justifies the necessity for developing models directly from soil exposure systems.

It is noteworthy to mention the work on the development of empirical bioavailability models for soils that are used in EU regulatory settings (ECB, 2009). These models were

developed after it became clear that terrestrial biotic ligand models for metals like Cu, Ni, and Zn were not readily feasible. For example, Smolders et al. (2009) showed that effects of Ni (and other metals) on soil organisms were best predicted by empirical relationships based on soil CEC and pH, and not on concentrations or activities of metal in relevant soil phases, such as porewater. This does not necessarily contradict the concept that the free metal ion in (soil) solution is the directly available and toxic species. This is readily explained by the BLM theory, which states that free ions dominate toxicity while cation competition will modify toxicity. For instance, in a recent study by Ardestani and Van Gestel (2013), it was found that soil pH had surprisingly little effect on Cd uptake in a springtail species (with a porewater-related uptake route), while there was a strong increase of Cd availability in the porewater with decreasing soil pH. The absence of a pH effect on Cd uptake kinetics could be explained from processes at the level of proton competition with Cd, which occurred at the level of porewater and were not visible when looking at the level of the soil. These aspects should be borne in mind when looking at different conclusions drawn respectively from empirical relationships for linking toxicity to soil properties and mechanistically underpinned models for linking toxicity to porewater chemistry (e.g., the Freundlich-type model).

6.3 Cross-species extrapolation

In order to generalize the developed bioavailability models within a regulatory framework, it is important to assess how variable the effects of porewater chemistry parameters on metal toxicity are across different soil species. Because it is unrealistic to develop BLM-based bioavailability models for all soil species, the earthworms were used as representatives for a larger group of major soil macrofauna. In Chapter II and Chapter III, we investigated whether the bioavailability model developed for one earthworm species could be extrapolated to another earthworm species. We further evaluated whether this model could be extrapolated to other soil species with different toxicological endpoints, based on literature data. Toxicity-modifying factors (e.g., H^+ for Cu toxicity, Mg^{2+} for Ni toxicity) were the same for different earthworm species, providing qualitative evidence for the option of extrapolating the model concept across species. A pH dependence of Cu toxicity was further found for soil invertebrates, plants, and microbial processes in a range of European soils. This confirms the applicability of the developed model and suggests that earthworm species well represent other species, especially those species that are in close contact with porewater or have porewater-mediated uptake of metal. Due to the fact that the effect of pH and other cations on metal toxicity differed for different species (as reflected by the model coefficients α and β_i, see Table 2.3 and Equations 3-8 and 3-9), quantitative extrapolation of the model is difficult. Based on our findings, a generalized model framework (i.e., $\log LC50\{M^{2+}\} = \alpha\ pH - \sum \beta_i\{C_i^{z+}\} + \gamma$) with separate coefficients for each model parameter can be created to predict the effect concentrations of metals for different species and different toxicological endpoints.

The topic of Chapter IV deals with important issues of differences in metal accumulation and sensitivity between earthworm species. These topics are worth to study, because they are connected with the extrapolation of results of ecotoxicological tests conducted mainly on *Eisenia fetida* (the so-called "white mouse" among earthworm species).

Expect for the artificially cultured species *E. fetida*, two field-relevant species *L. rubellus* and *A. longa* were tested. Metal accumulation patterns and sensitivity varied largely among the different earthworm species (*E. fetida*, *L. rubellus*, and *A. longa*), although these species belong to the same class (Oligochaeta). This indicates that the taxonomy is not an inherently informative descriptor for metal effects. Some studies have suggested that phylogenetically related aggregations of certain traits could provide a useful and alternative description of species-specific responses (Barnett et al., 2007; Rubach et al, 2010). In our study, the use of traits instead of species improved our understanding of earthworm responses to metal stress, providing the possibility for intraspecies and interspecies extrapolation. Most likely more than one or two traits determined species-specific accumulation and toxicity of metals in earthworms. The use of the traits-based approach for quantitative extrapolation of accumulation and toxicity data across species still needs research efforts. The traits-based approach has been widely used in ecological and evolutionary research (Violle et al., 2007). Challenges facing the future development of traits-based approaches in ecotoxicology are met in selecting relevant traits, quantitatively integrating these traits, and linking traits with mechanistic effect models. In these respects, qualitative comparative analysis (Caren and Panofsky, 2005) could be adopted to provide indications on which traits are of most interest in explaining variations among species. Alternatively, the trait frequency analysis and multivariate analysis (De Lange et al., 2013) could be suitable methods for quantitatively describing the relation between traits and earthworm responses.

6.4 Interpretation of mixture effects

As metals are often introduced into the environment as mixtures, assessment of mixture effects is extremely relevant. The classical way of evaluating mixture toxicity is to use either the concentration addition model or the effect addition model as a reference. Interpretation of the observed interactions is therefore often limited to overall antagonism or synergism. In this thesis, more complex patterns such as dose-ratio dependent and dose-level dependent deviations from the reference model were quantified by the MIXTOX model for a binary mixture of Cd and Zn in one soil type (Chapter V). A broad range of concentration combinations (239 combinations) was employed, assuring the identification of possible deviations. Only a limited number of replicates were conducted in order to allow covering as much as possible the exposure range of both metals. Therefore, effort was devoted to the number of combinations at the expense of the number of replicates. This will not bias the interpretation of obtained results because the concentration spacing in our study is generally small and the response surface analysis for determining interactions is based on a regression model (Jonker et al., 2004, 2005). Besides, the repeated tests showed favorable reproducibility (LC50 values of duplicates were within 10% deviation).

With respect to the mixture effects, a general conclusion on interaction patterns and interaction levels cannot be drawn due to the specificity of interactions in terms of metal combination and soil being tested. In this thesis, we found that interactions of Cd and Zn were mainly antagonistic, and the magnitude of antagonism depended on both the relative concentration of each metal and the concentration magnitudes. Baas et al. (2007) investigated the lethal effect of a binary mixture of Cu and Cd on the springtail *Folsomia candida* and

found that interactions of Cu and Cd were mainly synergistic. In our case, interactions of Cd and Zn occurred at the organism level but not at the exposure or soil level, while Van Gestel and Hensbergen (1997) observed physicochemical interactions of Cd and Zn when exposing *Folsomia candida* to an artificial soil. In a study by Sdepanian (2010), mixture interactions (Cu and Zn, Cd and Zn) in four soils were suggested to be related to a greater extent to the speciation effect (i.e., interactions at the exposure level) than to the interactions at the organism level. While an integrated interpretation of mixture effects is not possible, the above findings clearly show the importance of identifying interactions at relevant levels and of incorporating bioavailability effects in modelling mixture toxicity. This can be especially helpful to explain differences in interaction patterns that occur between different soil types.

Different expressions of exposure (total, porewater, and $CaCl_2$-extractable metal concentrations) yielded similar interaction patterns for Cd and Zn mixtures. This may be because of the fact that only one sandy loam soil was tested and strong correlations existed between total concentrations, porewater concentrations and $CaCl_2$-extractable concentrations (see Table 5.2). It is therefore suggested that more soil types should be tested for the purpose of comparison and extrapolation. To get the full picture of bioavailability effects on mixture toxicity in soil, metal speciation, competition and complexation, and interactions with organisms should be explicitly considered. Mixture modelling might be expanded with mechanistic bioavailability models such as the BLM and the electrostatic toxicity model (Wang et al., 2008) to optimize accurate effect prediction. In mixtures, metals may interact with each other to different extents (Vijver et al., 2010). Assuming that competition acts as a mechanism for metal mixture interactions (Niyogi and Wood, 2004), the use of BLM-based approaches for interpreting mixture effects is suitable. Jho et al. (2011) extended the BLM to predict the toxicity of Cd and Pb at different concentrations of Ca^{2+} using single metal toxicity data, and obtained good agreement between observed and predicted effects. The electrostatic toxicity model assumes that metal uptake and toxicity are determined by the ion activity at the surface of the cell membrane. Cations (e.g., Ca^{2+}, Mg^{2+}, and H^{+}) in the bulk solution can reduce the negativity of the electrical potential at the surface of the cell membrane by charge screening and ionic binding (Kinraide et al., 1998; Wang et al., 2011b), which in turn reduce toxic metal ion activities at the surface of the cell membrane. This modelling approach allows incorporating the effects of various cations simultaneously in modelling mixture toxicity and may provide mechanistic insights (in addition to competitive binding) into mixture interactions.

6.5 Implications for ecological risk assessment and soil quality criteria derivation

Ecological risk assessments for metals in the environment include two aspects: prospective and retrospective (Brock et al., 2013; Posthuma et al., 2008). Prospective risk assessment concerns the evaluation of the probability of adverse effects of metal exposure in the environment before the marketing, release, and use of the metal. It is a predictive approach which often uses toxicity data from standard laboratory toxicity tests to derive critical limits of metals in the environment (Van Gestel, 2012). Retrospective ecological risk assessment concerns the adverse impact of existing metals in the environment. It is a diagnostic approach which makes use of measured exposure concentrations or biological

effects in the exposed environment and enables setting priorities for remediation. The outcome of toxicity tests in this PhD thesis can be used to establish critical limits of metals in soil, which can then be compared with the measured or predicted exposure levels to determine the potential risk for metal contaminated sites. The developed bioavailability models (Chapter II and Chapter III) provide a mechanistic framework to normalize Cu, Cd, and Ni toxicities to a range of terrestrial species among different soils. These models directly link the toxicological endpoints to soil porewater chemistry, which in turn is linked to soil properties. The ability of the parameterized bioavailability model to predict toxicity on the basis of total soil metal is useful for standard-setting and risk assessment purposes. To set critical loads of metals for soils, WHAM VI can be used to calculate the total metal concentration in equilibrium with critical free metal ion activities (e.g., LC50$\{Cu^{2+}\}$) in soil porewater. An example can be found in the BLM work of Thakali et al. (2006a). Uncertainties in calculated critical loads for a large part lie in the calculation of solution speciation. For the soil solution where natural dissolved organic matter (DOM) is present, it is often needed to specify the ratio of active humic acid to fulvic acid, and the transfer coefficient between DOM and dissolved organic carbon (DOC) (usually, DOM = 2 DOC) as the physicochemical characteristics and thermodynamic data (such as reactions with metal ions, stability constants, and the number of reactive sites) of DOM are not clearly understood (Tipping et al., 2003; Dudal and Gérard, 2004). In addition to the mechanistic speciation calculation, the developed bioavailability models could readily be coupled to an empirical partitioning model to enable the link to total soil metal concentration to be made. For example, Lofts et al (2004) proposed that free metal ion activity ($\{M^{2+}\}$, mol/L) in soil solution can be predicted based on total soil metal ($[M]_{soil}$, mg/kg), soil pH and soil organic carbon content (OC, %) with the following equation:

$$\log(\{M^{2+}\}) = a \log([M]_{soil}) + b\,pH + c \log(OC) + d \quad (6\text{-}1)$$

where a, b, c and d are constants. With this relationship, we can easily relate, for example, the obtained critical free Cu^{2+} activity to critical total soil Cu concentration and other commonly measureable soil properties.

The current approaches for setting soil quality criteria for metals (e.g., NOECs) are strongly dependent on critical values, for example EC50s and LC50s of metals, obtained at standardized conditions. For derivation of these critical values, basic information on fate, exposure, and toxicity is investigated. However, the derivation following precautionary principles must be conservative as the critical values do not consider site-specific conditions. Exceeding the critical values does not necessarily imply a risk. This disadvantage can be overcome by using the BLM-based bioavailability models to link critical values to varying environmental conditions (e.g., pH, competing cations, and DOC). Legislators and regulators are moving forward to incorporate measures of bioavailability into regulations regarding water and soil quality. The BLM approach has been adopted by the USEPA to set aquatic life criteria for copper (USEPA, 2007). It has also been proposed for use in European Union risk assessments (Ahlf et al., 2009). In our study, the developed bioavailability models comply with the BLM theory. Their data requirements are small compared with BLMs and only routine parameters are needed as model inputs. Within regulatory frameworks, these models can help to remove the influence of test-specific abiotic conditions in the ecotoxicity database and to derive appropriate soil quality criteria.

Improving the accuracy of ecological risk assessment for metals is a primary benefit of bioavailability analysis (Ehlers and Luthy, 2003). A tiered approach which incorporates bioavailability for performing risk assessment of contaminated sites is recommended by Kördel et al. (2013). The different steps are modified and illustrated in Figure 6.1. As a start, historical examination and data gathering are needed (Step 1). If a metal contamination is suspected, soil sampling and analysis of the expected metal are performed (Step 2). A further bioavailability analysis must follow if trigger values (based primarily on total contents in current regulations) are exceeded (Step 3). The bioavailability analysis involves the determination of metal-specific exposure for specific objectives of protection and measurement of relevant bioavailability parameters (e.g., pH, competing cations). The modelling approaches developed in this thesis (Freundlich-type model and free ion approach) provide a more accurate characterization of bioavailability than traditional chemical extraction methods or empirical relationships, and these approaches thus fit well into step 3. Exceeding the trigger values corrected for bioavailability indicates a risk for a specific site. In this case, appropriate measures such as remediation or securing should be implemented.

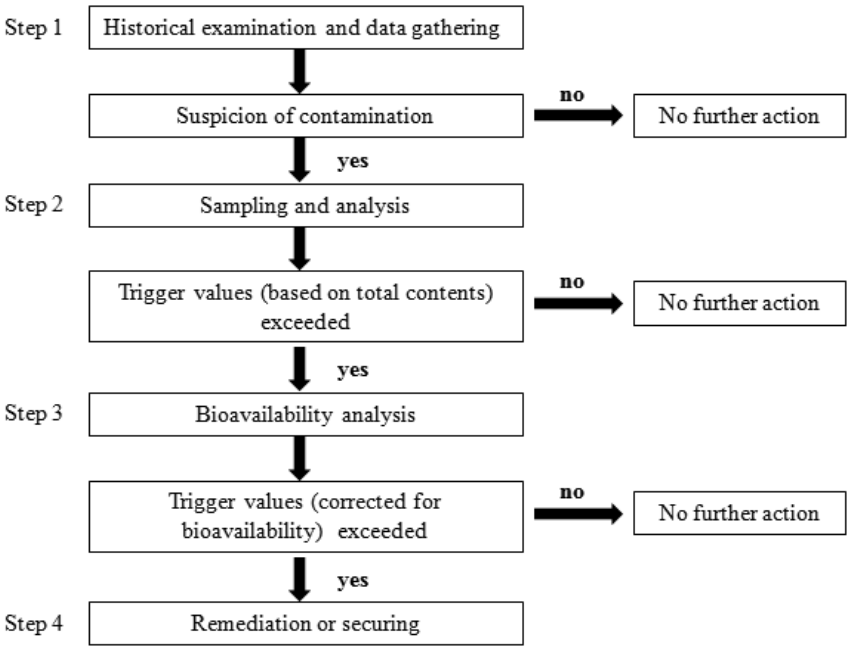

Figure 6.1 Incorporation of bioavailability in risk assessment of contaminated sites (adapted from Kördel et al., 2013, slightly modified)

Despite the fact that metals are commonly present as mixtures in the environment, mixture toxicity is not explicitly taken into account when evaluating the risks posed by metals in soil. Uncertainty factors (usually from 10 to 1000) are commonly used in risk assessment to deal with statistical uncertainties in the estimation of NOECs, biological variance, intra- and inter-species extrapolations, and extrapolation from acute to chronic effects (Posthuma et

al., 2008). In the common practice of soil protection, it is sometimes argued that uncertainty factors already cover the possibility of mixture effects. But a specific mixture assessment factor is currently not employed in chemical-by-chemical risk assessment (Kortenkamp et al., 2009). It still needs to be clarified how large a mixture assessment factor would need to be to take proper account of mixture effects. In the practice of chemicals legislation, even the new European chemical legislation REACH focuses almost exclusively on the assessment of individual chemicals. Under this legislation, industry takes the responsibility for demonstrating the risks of the manufacture, use, and disposal of chemicals (Lahl and Hawxwell, 2006). Industry thus only cares about the limited number of individual chemicals they produce and thus ignore mixture effects in general. Our case study showed that interactions of Cd and Zn at the organism level significantly influenced the observed toxicity to earthworms. For effective and accurate risk assessment of metal mixtures it is required to have appropriate models or tools that enable the prediction of mixture effects, which covers both simple and complex mixtures and incorporates mixture interactions. A general tiered outline for the predictive ecotoxicological risk assessment of chemical mixtures is proposed by Backhaus and Faust (2012). Since the mode of action and interactions are not clearly understood, they suggest a sequential application of CA and IA in two tiers. The first tier uses exclusively CA as a basis for the preliminary risk assessment of the mixtures of concern, as mixture toxicities higher than predicted by CA are rare findings. Only if there are still indications for a potential risk, the second tier commences. This tier takes IA into consideration. Detailed information on this method can be found in the cited literature. As mentioned before (paragraph 1.4 of Chapter I), CA as well as IA assumes a non-interaction between the mixture components. Interactions may lead to either increased or decreased toxicity. Such cases cannot be properly handled in a generalized risk assessment scheme. The type and magnitude of interaction can vary because of its dependence on dose ratio and dose level as shown in our cases (Chapter IV). This raises questions about how these detailed aspects should be taken into account in the context of risk assessment and standard setting for a ‘cocktail’ of metals. With current scientific knowledge, it is not yet possible to predict responses to mixture effects in individual cases; at best this is possible only in terms of general response patterns (Vijver et al., 2011). Nevertheless, the assumption of additivity provides a conservative prediction of mixture toxicity, because metals act either additively or antagonistically in more than 70% of all cases as reviewed by Norwood et al. (2003) and Vijver et al. (2010; 2011). This means that mixture toxicity will be either predicted correctly or overpredicted. From a regulatory perspective, such a conservative approach is to be favored in a generic risk assessment scheme. It is only the exceptional cases (synergistic effects) that require further considerations and additional approaches.

6.6 Future outlook and recommendations

The findings of this thesis present a clear indication of the importance of bioavailability when modelling metal toxicity in soil. While the bioavailability models were developed on the basis of toxicity experiments performed in different soils covering a wide range of soil properties, the future use of these models may be limited to the range in soil properties for which they were derived. Nevertheless, we have reasons to be optimistic about regulatory

application of the developed bioavailability models since they provide a mechanistic framework and can be easily modified to incorporate the effects of other toxicity-modifying factors. A series of validation studies and meta-analysis using our model concepts are encouraged to be done in future. Besides, it would be possible to apply our models to geographically oriented databases in order to do GIS-mapping of the site-specific risks of metals on a large scale, as long as the basic soil information (i.e., porewater chemistry) required by the models is available.

Various single-species screening tests are often employed to detect possible harmful effects of metals in soil. In this thesis, earthworms were selected as the test species as they are the dominant soil macrofauna and play a vital role in soil functioning. However, numerous species and a complex food web are exposed in the soil environment. Therefore, a battery of toxicity tests should be used to evaluate the effects on different trophic levels, as well as acute, chronic, and next-generation effects. Until now, very few research efforts have been made to determine the minimum battery of tests needed. To obtain a balanced battery of tests, it is recommended that a series of important criteria for selecting representative species among others need to be met, such as having different life-histories and exposure routes, and belonging to different functional and taxonomic groups. Besides, the selection of a battery of tests should be tailored to specific protection objectives.

Most of the bioavailability models are developed at a fixed time point, while the toxicodynamic part of the model is simply to relate the bioavailable faction (e.g., *f* value in BLM) to a toxicological endpoint (e.g., LC50 or EC50). This is also the case in our study. However, it has been recognized that bioavailability is not a static but a dynamic phenomenon (see Figure 1.1). For a better understanding of metal bioavailability, the underlying toxicokinetic and toxicodynamic processes deserve further investigation. This kind of research will enable the extrapolation of metal effects in the course of time and from soil organisms to higher organisms.

Our study distinguished the interactions of Cd and Zn at the exposure level from those at the organism level. However, the interactions of metal mixtures observed in this study yield little information about the internal processes involved. In fact, mechanistic pathways of metals inside the organism are poorly known. We therefore recommend that future studies should investigate the mechanisms of mixture interaction and identify the principles of combined toxicity for developing predictive models. Potential topics include, for example, considering the distribution and detoxification mechanisms (i.e., toxicological bioavailability) of one metal in the presence of other metals, and linking mixture effects to the expressions of specific proteins or genes (proteomics and genomics tools).

The issues of metal bioavailability and mixture toxicity have increasingly gained attention on the agenda of risk assessors and risk managers. The findings in this thesis therefore well fit into the broader picture of soil metal risk assessment. Despite the lack of mechanistic understanding of internal processes, the awareness of bioavailability and mixture toxicity has triggered the development of a series of chemical and modelling approaches to normalize the toxicity data in different environmental conditions, and thus has greatly improved risk assessment and standard setting. In case of remediation, environmental regulators are able to choose the sites for remediation where the actual risks are greater rather than blindly choosing the polluted localities where the total metal concentrations are

relatively high. This also avoids unnecessarily strict requirements for cleanup and thus avoids expenditure of funds that could be better used to remediate additional areas. We recommend continuing to discuss and question the relevance of the current approaches for risk assessment and decision making, and to update them according to the available tools and scientific knowledge on bioavailability and mixture toxicity.

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Summary

Metals in soils can pose a serious threat to soil dwelling organisms, plants, and human beings. A major uncertainty in terrestrial ecological risk assessment for metals is the integrated effect of the physicochemical properties of soil on toxicity and how this allows for extrapolation of toxicity data across soils. The recognition that soil type is an important factor that determines metal toxicity, goes along with the increasing insight into bioavailability. There is a growing consensus that only a proportion of the total amount of metal in soil is available for uptake by organisms and subsequently induces toxic effects. Development of mechanistically underpinned approaches for explaining and predicting availability effects on metal toxicity has been the subject of many research efforts in terrestrial ecotoxicology.

The biotic ligand model is often used as the state-of-the-art approach to quantify the effect of metal availability on metal toxicity in aquatic systems. The biotic ligand model is a synthesis of decades of work on metal speciation, accumulation, toxicity and physiology. The BLM combines two major aspects that affect toxicity: metal speciation and cation competition. Toxicity is assumed to be proportional to the fraction of biotic ligand sites occupied by the free metal ions and competitive binding of other cations with metal ions to the biotic ligand sites can alleviate toxicity. These aspects have been used to explain, why toxicity to freshwater organisms in many cases varied greatly with water chemistry parameters such as pH, hardness, and DOC. The BLM concept is shown to be applicable to a wide range of aquatic organisms, for example fish, daphnia, and algae. In this thesis, we explored the applicability of BLM theory for soil organisms, with a special focus on earthworms (Chapter II and Chapter III). We have adopted the equilibrium partitioning theory and the porewater hypothesis in order to enable linking metal toxicity to porewater chemistry and to apply the BLM theory to soil toxicity data. It is assumed that there is a dynamic equilibrium between metals in the soil solid phase and the soil porewater and that free metal ions in soil porewater are the main toxic species for soil organisms while the base cations can mitigate toxicity through competition for binding to the biotic ligand sites.

In Chapter II, a multicomponent Freundlich model, complying with the basic assumptions of the BLM, was developed to describe the variations in Cu toxicity to earthworms (*Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*) in a range of soils of varying properties. This model has some conceptual and practical advantages over the BLM as it requires fewer parameters than the BLM and considers site heterogeneity. Only H^+ but not other cations (K^+, Ca^{2+}, Na^+, and Mg^{2+}) in soil porewater exerted a significant role against Cu^{2+} toxicity to earthworms. The Freundlich-type model in which the protective effects of H^+ were included, explained 84%, 94%, and 96% of the variation in LC50$\{Cu^{2+}\}$ for *L. rubellus*, *A. longa* and *E. fetida*, respectively. Predicted values of LC50$\{Cu^{2+}\}$ never differed by more than a factor of 2 from the observed values. External validation of the model showed a similar level of precision, even though toxicity data for other soil organisms and other endpoints were used. Our findings showed the possibility of extrapolating the developed models for one earthworm species to another earthworm species, and even to other soil organisms with different toxicological endpoints.

In Chapter III, we investigated the effects of Cd and Ni on the survival of earthworms (*L. rubellus* and *A. longa*) in soils. Based on empirical studies and the BLM theory, the free ion

approach was proposed as an alternative method to predict Cd and Ni toxicity to earthworms. For the two tested earthworm species, no significant influence of cations (H^+, K^+, Ca^{2+}, Na^+, and Mg^{2+}) on Cd^{2+} toxicity was seen, while Mg^{2+} was found to significantly alleviate Ni^{2+} toxicity. The free ion activity model, which is a special case of the free ion approach, sufficiently described the variability in Cd^{2+} toxicity across soils but failed in predicting Ni^{2+} toxicity. The free ion approach, in which the protective effects of Mg^{2+} were incorporated, explained 89% and 84% of the variations in LC50{Ni^{2+}} for *L. rubellus* and *A. longa*, respectively. Prediction errors were within a factor of 2. There was a lack of consistent effects from the presence of possible competing cations for different metals (i.e., H^+ for Cu^{2+}, Mg^{2+} for Ni^{2+}, and none for Cd^{2+}). We therefore suggest that metal toxicity to earthworms needs to be evaluated on a metal-specific basis.

Another uncertainty in ecological risk assessment is species-specific responses. It has become apparent that the taxonomy-based approach is inappropriate in estimating metal toxicity. This is because taxonomically related species do not necessarily show similar sensitivity to metals. Instead, each species may possess different combinations of traits (characteristics) to deal with metal stress.

In Chapter IV, accumulation and toxicity of Cu, Cd, Ni, and Zn in three earthworm species (*L. rubellus*, *A. longa* and *E. fetida*) were examined. At the same exposure concentration, internal concentrations followed the order: *L. rubellus* > *E. fetida* > *A. longa* for Cu and Ni, *L. rubellus* ≈ *E. fetida* ≈ *A. longa* for Cd, and *L. rubellus* > *A. longa* > *E. fetida* for Zn. The concentrations of Cu, Cd, and Zn in *E. fetida* generally levelled off at high exposure concentrations but not for the other two species. *A. longa* showed a high capability of regulating internal Ni concentrations. For all the four metals tested *L. rubellus* was the most sensitive species, followed by *A. longa* and E. *fetida*. Based on traits theory, a set of traits (such as soil habitat, mobility, ratio of surface area to mass) can be related to the metal accumulation ranking of the earthworm species tested. Differences in sensitivity between species might be explained using the following traits: soil habitat, calcium glands activity, ratio of surface area to mass, and immune-competent cells. However, implementing the traits theory for quantitatively explaining all the observed accumulation patterns and sensitivity of different earthworm species was shown to be difficult. More research is needed in this respect to build up solid relationships between species-specific responses and traits, enabling cross-species extrapolation of accumulation and toxicity data.

In soil, metals are typically present as mixtures because of their natural origin as mixtures in parent rocks and because of diverse pollution sources. Interactions of mixture components can occur at different toxicological levels, making it difficult to predict toxicity of metal mixtures. Classical mixture models (CA or IA) with additivity as the basis ignore these interactions. This may cause overestimation or underestimation of the actual risks.

In Chapter V, individual and binary mixture effects of Cd and Zn on the survival of the earthworm *Aporrectodea caliginosa* were investigated. An increase in Cd concentration decreased Zn toxicity. Similarly, the addition of Zn over a certain concentration range (100 to 1000 mg/kg) decreased Cd toxicity. Beyond a critical Zn concentration of around 1000 mg/kg, Cd toxicity dramatically increased. The partitioning of Cd and Zn between the soil solid phase and the porewater was affected neither by the concentrations nor by the presence of the other metal, suggesting no interactions at the exposure level. Interactions of Cd and Zn

occurred at the organism level, limiting the predictive ability of the simple concentration addition model. MIXTOX modelling showed that mixture interactions were mainly antagonistic and the magnitude of antagonism depended on the relative concentrations of Cd and Zn, and on the concentration magnitudes for the whole ranges of concentrations tested. These findings highlight the importance of identifying the relative influence of various interactions from external exposure to internal assimilation in assessing metal toxicity.

To conclude, our studies present a clear indication of the importance of bioavailability when modelling metal toxicity in soil and suggest the applicability of the BLM theory to soil organisms. The developed bioavailability models (the Freundlich-type model and the free ion approach) in this thesis provide a mechanistic framework for linking soil porewater chemistry to metal toxicity in soils. The traits-based approaches assist in explaining differences in metal accumulation and toxicity between earthworm species. Integration of effect models and traits-based approaches may offer a means to facilitate extrapolation of accumulation and toxicity data across species. With respect to the mixture effects, our study clearly shows the importance of identifying interactions at relevant levels and of incorporating bioavailability effects in modelling mixture toxicity. This can be especially helpful to explain differences in interaction patterns that occur between different soil types. Ultimately, the findings in this thesis well fit into the broader picture of soil metal risk assessment.

Samenvatting

Metalen in de bodem kunnen een ernstige bedreiging voor het bodemleven, planten en dieren, en mogelijk zelfs voor mensen zijn. Een belangrijke onzekerheid in terrestrische ecologische risicobeoordeling voor metalen is het effect van de fysisch-chemische eigenschappen van de bodem op de toxiciteit. Deze eigenschappen beïnvloeden de vertaling van toxiciteitsgegevens over verschillende bodemtypen. De erkenning dat bodemtype een belangrijke factor is in het beschrijven van ecotoxicologische effecten, gaat samen met een toenemende inzicht in de factoren die de biobeschikbaarheid van een metaal bepalen. Er is een groeiende consensus dat slechts een deel van het in de bodem aanwezige metaal, beschikbaar is voor opname door organismen en dat slechts dit deel de toxische effecten indiceert. Ontwikkeling van mechanistisch onderbouwde benaderingen voor het verklaren en voorspellen van biologische effecten van de aanwezigheid van metalen in de bodem is het onderwerp van veel onderzoek in terrestrische ecotoxicologie geweest.

Het biotische ligand model wordt vaak gebruikt als de state-of- the-art aanpak om het effect van de biobeschikbaarheid op de toxiciteit van metalen in aquatische systemen te kwantificeren. Het biotische ligand model is een synthese van tientallen jaren werk op het gebied van metaalspeciatie, accumulatie, toxiciteit en fysiologie. De BLM combineert twee belangrijke aspecten die de toxiciteit beïnvloeden: metaalspeciatie en kation concurrentie. De fractie van de plaatsen van het biotische ligand die bezet worden door de vrije metaalionen beschrijft de toxiciteit die optreedt. De andere kationen hebben een competitieve invloed met de metaalionen op de biotische ligand sites, wat kan leiden tot vermindering van de toxiciteit bij een gelijkblijvend metaalgehalte. Deze factoren worden gebruikt om te verklaren waarom in veel gevallen de toxiciteit voor zoetwater organismen sterk varieert met chemische parameters zoals pH, hardheid, en DOC gehalte. De BLM theorie is aantoonbaar toepasbaar op een breed scala aan waterorganismen, waaronder bijvoorbeeld vissen, evertebraten en algen. In dit proefschrift hebben we de toepasbaarheid van de BLM theorie onderzocht voor bodemorganismen, met de focus op regenwormen (hoofdstuk II en hoofdstuk III). Om de BLM theorie te gebruiken voor het voorspellen van de effecten van metalen op bodemorganismen, hebben we de evenwichtspartitie-theorie en de poriewater-hypothese vanuit de aquatische ecotoxicologie geadopteerd. Dit wil zeggen dat we aannemen dat er een dynamisch evenwicht is tussen metalen in de vaste fase van de bodem en de metalen in het poriewater. Daarbij is aangenomen dat de vrije metaalionen in het poriewater de toxiciteit op het bodemleven bepalen. En dat de aanwezige kationen in het poriewater, de metaal toxiciteit op het bodemleven verminderen door hun competitieve binding aan de biotische ligand van de organismen.

In hoofdstuk II is een multicomponent Freundlich model gebruikt om de variaties in Cu toxiciteit te kwantificeren voor de regenwormen soorten (*Lumbricus rubellus*, *Aporrectodea longa*, en *Eisenia fetida*), welke blootgesteld waren in een reeks van bodems met verschillende eigenschappen. Het multicomponenten Freundlich model voldoet in de basis aan de uitgangspunten van de BLM. Het Freundlich model heeft een aantal conceptuele en praktische voordelen ten opzichte van de BLM, allereerst omdat het minder parameters vereist dan de BLM terwijl het model daarnaast de heterogeniteit van de biotische sites meeneemt. Onze resultaten laten zien dat alleen H^+, en dus niet de andere kationen (K^+, Ca^{2+},

Na^+ en Mg^{2+}) in het poriewater, in competitie waren met koper. De pH speelt daarmee een belangrijke rol in het beschrijven van de Cu^{2+} toxiciteit voor regenwormen. Het Freundlich model kon met het meenemen van de H^+-competitie, respectievelijk 84 , 94 en 96 % van de variaties in LC50{Cu^{2+}} verklaren voor *L. rubellus*, *A. longa* en *E. fetida*. De voorspelde LC50{Cu^{2+}} waarden verschilden nooit meer dan een factor 2 van de gemeten waarden. Externe validatie van het model toonde een vergelijkbaar niveau van precisie, zelfs als er toxiciteitdata voor andere bodemorganismen en andere eindpunten werden gebruikt. Onze bevindingen toonden de extrapolatie mogelijkheid van de ontwikkelde modellen voor één regenworm soort naar andere soorten regenwormen aan. Het model kon zelfs gevalideerd worden naar andere bodemorganismen met verschillende toxicologische eindpunten.

In hoofdstuk III onderzochten we de effecten van Cd en Ni op de overleving van twee soorten regenwormen (*L. rubellus* en *A. longa*) in de bodem. Uit de resultaten van het empirische onderzoek in combinatie met de BLM theorie werd de vrije ion aanpak voorgesteld als een adequate alternatieve methode om Cd en Ni toxiciteit te voorspellen voor regenwormen . Voor de twee geteste regenwormen werd geen invloed van kationen (H^+, K^+, Ca^{2+}, Na^+ en Mg^{2+}) op de Cd^{2+} toxiciteit waargenomen, terwijl het kation Mg^{2+} wel de Ni^{2+} toxiciteit significant verminderde. Een iets andere benadering, genaamd het `vrije ion activiteit` model beschreef de variabiliteit in Cd^{2+} toxiciteit in de bodem beter maar kon niet afdoende de Ni^{2+} toxiciteit voorspellen. Het `vrije ion activiteit` model waarbij de beschermende effecten van Mg^{2+} werden opgenomen, verklaarde respectievelijk 89 en 84 % van de variaties in LC50{Ni^{2+}} waarden voor *L. rubellus* en *A. longa*. De voorspelling kon gemaakt worden binnen een factor 2. Voor ieder metaal dat we getest hadden, bleek een andere kation de competitieve binding op het biotische ligand te beïnvloeden en daarmee dus de toxiciteit. We concluderen daaruit dat de toxiciteit van metalen voor regenwormen moet worden beoordeeld op een metaal-specifieke basis.

Een andere onzekerheid in de ecologische risicobeoordeling is de soort-specifieke response. Het is duidelijk geworden dat een op taxonomie gebaseerde benadering de toxiciteit van metalen slecht inschat. Dit komt omdat taxonomisch verwante soorten niet noodzakelijkerwijs een gelijkwaardige gevoeligheid voor metalen hebben. In plaats daarvan kan elke soort verschillende combinaties van eigenschappen (organisme kenmerken) hebben die de metaal stress kunnen beïnvloeden.

In hoofdstuk IV is de accumulatie en toxiciteit van Cu, Cd, Ni en Zn voor drie soorten regenwormen (*L. rubellus*, *A. longa* en *E. fetida*) onderzocht. Wanneer de regenworm soorten zijn blootgesteld aan gelijke metaal concentraties, is de hoogste interne van Cu en Ni concentratie te vinden in: *L. rubellus* > *E. fetida* > *A. longa*. Voor Cd is de volgorde als volgt: *L. rubellus* ≈ *E. fetida* ≈ *A. longa*. En voor Zn is de volgorde: *L. rubellus* > *A. longa* > *E. fetida*. De concentraties van Cu, Cd en Zn in *E. fetida* waren in het algemeen gestabiliseerd bij hoge blootstellings concentraties, maar niet voor de andere twee species. *A. longa* toonde een hoge capaciteit voor het reguleren van de interne Ni concentraties. *L. rubellus* was de meest gevoelige regenwormsoort voor alle geteste metalen, gevolgd door *A. longa* en *E. fetida*. Vanuit de theorie kunnen verschillen in metaalgevoeligheid worden verklaard vanuit de organisme kenmerken, waaronder de leefomgeving (habitat), mobiliteit van organismen, en hun oppervlak/massa ratio. Verschillen in metaal-gevoeligheid van de regenwormsoorten kan worden verklaard vanuit organisme kenmerken zoals habitat, activiteit van de calcium-

klieren, verhouding van oppervlak tot gewicht, en aanwezigheid van immuun-competente cellen. Het kwantitatief relateren van gemeten accumulatiepatronen en gevoeligheid van verschillende regenwormsoorten met de soort kenmerken bleek lastig te zijn en meer onderzoek is nodig in dit verband.

Metalen in de bodem komen voor als mengsels. De componenten in een mengsel kunnen op verschillende toxicologisch niveaus interacteren, waardoor het moeilijk is om de toxiciteit van metalen mengsels te voorspellen. Klassieke mengselmodellen (zogenaamde Concentration Addition (CA) of Independent Action (IA)) met additiviteit als basis negeren deze interacties. Deze afwijking van additiviteit kan overschatting of onderschatting van de werkelijke effecten veroorzaken.

In hoofdstuk V werden individuele en binaire mengseleffecten van Cd en Zn op de overleving van de regenworm *Aporrectodea caliginosa* onderzocht. Bij verhoogde Cd concentraties, verminderde de Zn toxiciteit. De toevoeging van Zn (tussen de 100 tot 1000 mg Zn/kg bodem) verminderde de Cd toxiciteit voor de regenworm. Boven een kritische Zn concentratie van ongeveer 1000 mg Zn/kg bodem, nam de Cd toxiciteit sterk toe. De verdeling van Cd en Zn tussen de vaste fase en het poriewater werd noch door de concentratie of de aanwezigheid van andere metalen beïnvloed. Dit suggereert dat er geen interacties van beide metalen zijn op het blootstellingniveau. Wel werden interacties van Cd en Zn op het organismeniveau gevonden. Deze interacties limiteerden het voorspellende vermogen van de eenvoudige additiemodellering zoals binnen de MIXTOX module wordt gehanteerd. De afwijking van additiviteit was voornamelijk richting antagonisme en de omvang van antagonisme fhangt af van zowel de relatieve concentraties van Cd en Zn, als van de gebruikte concentratiesniveau's. Deze bevindingen benadrukken het belang van het identificeren van de relatieve invloed van de verschillende interacties van externe blootstelling op de interne assimilatie bij de beoordeling van de toxiciteit van metalen.

Tot slot, onze studies laten duidelijk het belang van biobeschikbaarheid zien binnen de modellering van metaaltoxiciteit in de bodem. De resultaten laten zien dat de BLM theorie toepasbaar is voor bodemorganismen. De modellen (multicomponenten Freundlich model en het 'vrije ion activiteit' model) zoals in proefschrift ontwikkeld, bieden een mechanistisch basis voor het koppelen van de poriewaterchemie aan de metaal toxiciteit. De op organisme kenmerken gebaseerde benadering helpt bij het verklaren van verschillen in de accumulatie en toxiciteit tussen regenwormsoorten. Integratie van de verschillende benaderingen kan een middel zijn om tot extrapolatie van accumulatie en toxiciteit inschattingen over verschillende diersoorten te komen. Onze studie toont duidelijk het belang van het identificeren van interacties op relevante niveaus, waarna de biobeschikbaarheid ingepast kan worden binnen de toxiciteitsmodellen. Dit kan vooral zijn nut hebben bij het verklaren van de verschillen in interactiepatronen die aanwezig zijn bij verschillende grondsoorten. Uiteindelijk zijn de bevindingen in dit proefschrift goed toe te passen in het bredere beeld van de risicobeoordeling van metalen in de bodem.

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Hao Qiu
June 2014, Leiden, NL

Curriculum vitae

Hao Qiu was born on 22rd Feb. 1986 in Xinghua, Jiangsu, China. He studied in the Xinghua High School from 2001 to 2004. After passing the National University Entrance Examination in June 2004, he started his university education at the Department of Environmental Engineering, Nanjing University of Science and Technology, and graduated in 2008 with a bachelor degree. During that period, he carried out a six month internship on microbial degradation of polycyclic aromatic hydrocarbons for his bachelor dissertation in the group of Prof. Xin Jiang and Dr. Fang Wang in the Institute of Soil Science, Chinese Academy of Sciences. In 2010, He received his master's degree in Environmental Engineering from the School of Environmental Science and Technology, Sun Yat-sen University. In the Bioremediation group lead by Prof. Rongliang Qiu, his master research mainly focused on the evaluation of metal bioavailability in amended soils using the Diffusive Gradients in Thin Films technology. Following completion of the master thesis, he joined the Institute of Environmental Sciences, Leiden University as a PhD researcher. From 2010 to 2014, he undertook a PhD project on the development of a consistent methodology for assessment of the bioavailability and toxicity of metals in soil under the supervision of Prof. Willie Peijnenburg and Dr. Martina Vijver. After getting his PhD, he will continue his scientific research career.

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